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EXPLORING LYMPHOCYTE SUBSETS, AUTOANTIBODIES AND THE EFFECT OF B CELL TARGETED THERAPIES IN RHEUMATIC DISEASES

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Exploring lymphocyte subsets, autoantibodies and the effect of B cell targeted therapies in rheumatic diseases

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To my family and friends.

'All my life through, the new sights of Nature made me rejoice like a child.'

Marie Curie

ABSTRACT

Nowadays, around 100 different diseases sort under the umbrella of rheumatic diseases(1). They involve different mechanisms and symptoms engaging joints, tendons, ligaments, bones, muscles but also vital organs like the kidneys nervous system and circulation. The diagnoses is dependent on a combination of common clinical manifestations and laboratory findings such as the presence of specific antibodies that target self-antigen (2–4). Rheumatic disease evolves from a complex interplay between genetic, stochastic and environmental factors resulting in a loss of immune tolerance. B-cell targeting agents have proven useful in the treatment of several rheumatic diseases. Rituximab, a chimeric monoclonal antibody targeting CD20, is e.g. used in rheumatoid arthritis (RA) patients who do not respond to tumor necrosis factor (TNF) inhibitors and as off label-rescue treatment in systemic lupus erythematosus (SLE). Additionally, belimumab, a B cell activating factor (BAFF) inhibitor, is the first approved biological drug for SLE. Even though B-cell targeting therapies are commonly used, little is known about the effect of these therapies on disease associated B and T cell subsets, such as age/autoimmune-associated B cells (ABCs) and T follicular helper (Tfh) cells.

In **paper I,** we therefore studied the effect of these two subsets in response to rituximab treatment in SLE patients and found that ABCs are indeed influenced by the treatment while Tfh frequencies stayed similar to baseline. Additionally, we observed a decrease frequency of programmed cell death protein 1 (PD-1)^{high} CD4+ T cells.

In **paper II,** we investigated the effect of belimumab on circulating B and T cell phenotypes in SLE patients using mass cytometry and correlated them with clinical response. Belimumab had rapid effects on B cell subsets of earlier developmental stages such as naïve B cells while late B cell stages, such as memory or plasma cells, decreased later in a gradual manner or did not change upon treatment. Only early immunological changes correlated with disease improvement. High B cell counts at baseline were associated with late or non-responders.

Not all patients respond to B-cell targeting therapy, highlighting the heterogenicity of SLE. To develop novel therapies, a better understanding of the underlying mechanisms related to clinical phenotypes is needed.

Thus, in **paper III**, we explored the cytokine profile and the cellular composition in the synovial fluid of lupus arthritis patients, and we found elevated levels of IL-6 and IL-17A in the joint. Furthermore, we found an enrichment of Th17, peripheral helper T cells and EOMES/Granzyme A-expressing T cells in synovial fluid of SLE patients. All in all, indicating a potential role of Th17 cells in the pathogenesis of lupus. In contrast to lupus

arthritis, RA patients exhibit a more aggressive form of arthritis, especially in conjunction with anti-citrullinated protein autoantibodies (ACPA).

Therefore, in **paper IV,** we investigated the citrulline-specific B cell population in RA patients using an antigen-tetramer enrichment technology followed by single cell sequencing of the immunoglobulin genes. We discovered that the broad ACPA specificity in RA patients might develop from clonal expansion of a few B cell clones. Furthermore, monoclonal antibodies which originated from citrulline reactive B cells were multi-reactive and able to promote pain-like behavior and joint inflammation in mice.

Overall, this thesis explored immunological changes upon B-cell-targeting therapy and pathogenicity in joint inflammation in SLE and seropositive RA patients. We provided new understanding of B-cell targeting therapies on B and T cell subsets in SLE patients as well as the pathogenic mechanism which might be involved in lupus arthritis. Additionally, we examined the citrulline reactive B cell repertoire in RA patients and our data reveal their potential origin.

LIST OF SCIENTIFIC PAPERS

I. **Sippl N***, Faustini F*, Stålesen R, Svenungsson E, Chemin K, Gunnarsson I*, Malmström V*.
Rituximab therapy in systemic lupus erythematosus - lymphocyte phenotypes and clinical outcome.
Manuscript

II. Ramsköld D*, Parodis I*, Lakshmikanth T, **Sippl N**, Khademi M, Chen Y, Zickert A, Mikeš J, Achour A, Amara K, Piehl F, Brodin P, Gunnarsson I, Malmström V.
B cell alterations during BAFF inhibition with belimumab in SLE. *EBioMedicine. 2019 Feb;40:517-527*

III. **Sippl N,** Faustini F, Rönnelid J, Turcinov S, Chemin K, Gunnarsson I, Malmström V. **Arthritis in systemic lupus erythematosus is characterized by local IL-17A and IL-6 expression in synovial fluid.**
Clin Exp Immunol. 2021 Feb 11.

IV. Titcombe PJ, Wigerblad G, **Sippl N**, Zhang N, Shmagel AK, Sahlström P, Zhang Y, Barsness LO, Ghodke-Puranik Y, Baharpoor A, Hansson M, Israelsson L, Skriner K, Niewold TB, Klareskog L, Svensson CI, Amara K, Malmström V, Mueller DL**.**
Pathogenic Citrulline-Multispecific B Cell Receptor Clades in Rheumatoid Arthritis.
Arthritis Rheumatol. 2018 Dec;70(12):1933-1945.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

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Amara K, Clay E, Yeo L, Ramsköld D, Spengler J, **Sippl N**, Cameron JA, Israelsson L, Titcombe PJ, Grönwall C, Sahbudin I, Filer A, Raza K, Malmström V, Scheel-Toellner D.
B cells expressing the IgA receptor FcRL4 participate in the autoimmune response in patients with rheumatoid arthritis.
J Autoimmun. 2017 Jul;81:34-43.

Amara K, Clay E, Yeo L, Ramsköld D, Spengler J, **Sippl N,** Cameron J, Israelsson L, Titcombe PJ, Grönwall C, Sahbudin I, Filer A, Raza K, Malmström V, Scheel-Toellner D.
Immunoglobulin characteristics and RNAseq data of FcRL4+ B cells sorted from synovial fluid and tissue of patients with rheumatoid arthritis.
Data Brief. 2017 Jun 7;13:356-370.

CONTENTS

LIST OF ABBREVIATIONS

ABCs	Age-associated B cells / autoimmune-associated B cells
ACPA	Anti-citrullinated protein autoantibodies
APRIL	A proliferation-inducing ligand
BAFF	B-cell activation factor
BAFFR/BR3	B-cell-activating factor Receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
C1q	Complement component 1q
cAMP	Cyclic adenosine monophosphate
CBA	Cytokine bead array
CCP	Cyclic citrullinated peptides
CCR	CC Chemokine receptors
CD	Cluster of differentiation
CDR	Complementarity-determining regions
cDC	Classical dendritic cells
CEP1	Cyclic enolase peptide 1
Cfc	Circular citrullinated peptides from filaggrin
CRP	C reactive protein
CSF	Cerebrospinal fluid
CVID	Common variable immunodeficiencies
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
EOMES	Eomesodermin
EXPLORER	The Exploratory Phase II/III SLE Evaluation of Rituximab
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
FcRL5	Fc Receptor Like 5
FO	Follicular
GC	Germinal center
GZMA	Granzyme A

HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IRF	Interferon regulatory factors
LLDAS	Lupus low disease activity score
LN	Lupus nephritis
LN	Lymph node
LUNAR	Lupus Nephritis Assessment with Rituximab study
mAb	Monoclonal antibody
MHC	Major histocompatibility complex (
MS	Multiple sclerosis
MZ	Marginal Zone
NET	Neutrophil extracellular traps
NF-kappa B	Nuclear factor kappa B
NK	Natural killer
P.gingivalis	Porphyromonas (P.) gingivalis
PAD	Peptidylarginine deiminase
PBMC	Peripheral blood mononuclear cells
PC	Plasma cell
PC	Principal component analysis
PD	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PTPN22	protein tyrosine phosphatase non receptor type 22 gene
RA	Rheumatoid Arthritis
RA33	Ribonucleoprotein A2/B1
RF	Rheumatoid Factor
RNP	Ribonucleoprotein particle
ROCK2	Rho-associated protein kinase
RORγt	Related-orphan nuclear receptor γt
SA	streptavidin
SE	Shared epitope
SF	Synovial Fluid
SS	Sjögren's syndrome

SLE	Systemic Lupus erythematosus
SLEDAI	SLE Disease Activity Index
SNP	Single nucleotide polymorphism
SRI	SLE responder index
SSA/Ro	Anti–Sjögren's-syndrome-related antigen A autoantibodies
SSB/La	Anti–Sjögren's-syndrome-related antigen B autoantibodies
ST	Synovial tissue
T-bet	T-box transcription factor TBX21
TACI	T cell activator and calcium modulating ligand interactor
TCR	T cell receptor
TEMRA	Effector memory T cells re-expresses CD45RA
Tfh	T follicular helper
Th	T helper cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{PH}	T peripheral helper
Treg	T regulatory cells
tSNE	t-Distributed Stochastic Neighbour Embedding
VH	Variable heavy gene
β2GPI	β2 glycoprotein I

1 INTRODUCTION

Rheumatic diseases are initiated through a complex interplay between genetic and environmental risk factors and stochastic changes, resulting in a break of tolerance, autoantibody production and subsequently clinical symptoms (5). The disease diagnosis and therapy are linked to clinical manifestations and laboratory measures, such as the presence of specific autoantibodies. B cells have gained attention in the pathogenic role of rheumatic and other autoimmune diseases due to the success of B-cell targeting therapy. This thesis focuses on the two rheumatic diseases: systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Here, we explored the effect of two B-cell targeting therapeutic agents belimumab and rituximab on T and B cell phenotypes in SLE patients, as well as the underlying mechanisms of synovitis in SLE. Furthermore, we investigated the citrulline-specific B cells repertoire and origin in RA patients along with their contribution in the pathogenesis of RA.

1.1 RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) affects around 0.5-1% of the world population of all ethnicities with an onset of age between 40 and 70 and is more prevalent in women compared to men (6,7). RA is characterized by chronic inflammation of the synovial joints in which predominantly small joints in the digits and extremities are affected in a symmetrical pattern. However, larger joints, such as the knee, hip and shoulder, can also be involved (6) .The inflammation in the joint induces pain, tenderness and if not treated, can lead to disabilities and premature mortality (6). RA is classified by the presence of synovitis in at least one joint in the absence of other diagnostic alternatives, duration, location of the joint as well as laboratory findings, such as acute-phase reactants and RA-related autoantibodies (serology) (2). Two types of autoantibodies have been assoicated with RA and are nowadays used as diagnostic tools: rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) (2). The characterization and association of these autoantibodies with the disease are discussed in the following sections.

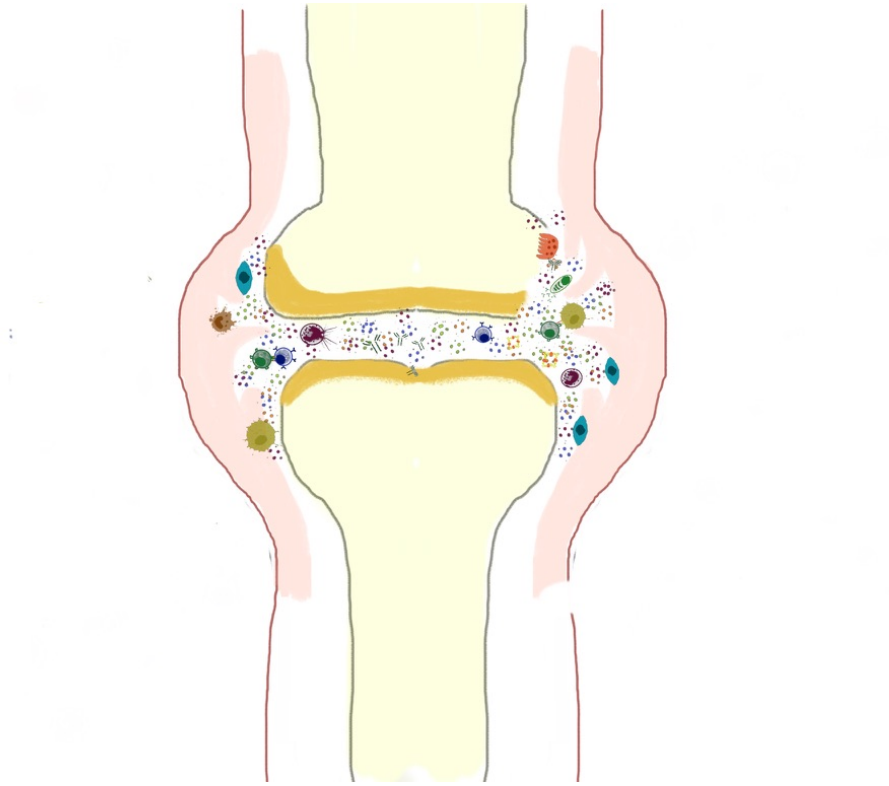


***Figure 1**: Joint inflammation in RA is characterized by immune cell infiltration, high levels of proinflammatory cytokines (IL-6, TNF), activation of osteoclasts and the presence of autoantibodies and immune completes.*

1.1.1 Rheumatic Factor (RF)

Rheumatoid Factor (RF) was discovered in 1940s and named after the association with RA (8). RF targets the fragment crystallizable region (Fc) region of the immunoglobulin (Ig) G and can be found in around 70% of RA patients (9). Although, RF is commonly used as diagnostic marker, it is rather unspecific, since it can also be detected in other autoimmune diseases, such as Sjögren syndrome (SS) and systemic lupus erythematosus (SLE), as well as in some healthy subjects and it increases with age (10). RF enhances immune complex clearance, by increasing B cell uptake of immune complexes and antigen presentation as well as complement fixation on immune complexes (11). Under normal conditions, RF is suggested to be produced by secondary immune responses.

In RA, increased titers, high affinity RF and the isotype IgA have been linked with bone erosion, severity and persistent disease (11–13). It has been proposed that RF is introduced from autoantibody formed immune complexes and contributes to the disease by forming immune complex and fixating complement which leads to tissue damage (11). Interestingly, RF derived from healthy individuals and RA patients exhibits similar characteristics. RF is of low affinity with moderate somatic maturation (14).

These results were replicated by Lu et al. together with the finding that RF positive B cells exhibit a specific transcriptional program which was associated with a rapid memory reactivation driven by multiple innate immune pathways such has Toll-like receptor (TLR) stimulation (15).

1.1.2 Anti-citrullinated protein antibodies (ACPA)

Antibodies directed towards citrullinated proteins are detected in 70% of RA patients and ACPA+RA patients have been shown to suffer from a more severe disease progression including aggressive bone erosion and enhanced radiological progression (16–18). In 2010, ACPAs were incorporated in the RA classification (2). For diagnostic purposes, ACPAs are primarily measured using cyclic citrullinated peptide (CCP) IgG ELISAs (19).

1.1.2.1 Citrullination a post-translational modification

Citrullination is a post-translational modification of proteins in which the positive charged amino acid arginine is converted into a neutral citrulline (20). Citrullination is irreversible and alters the conformation, function and half-life of a protein (21,22).

The modification occurs ubiquitously during cell differentiation, apoptosis and inflammation (21). Elevated levels of citrullinated proteins are present in several diseases such as multiple sclerosis, Alzheimer, inflammatory bowel disease and cancer, without the presence of ACPAs (23,24). This reaction is catalyzed by the enzyme peptidylarginine deiminase (PAD), which requires high levels of calcium. There are five different isotypes of the PAD enzyme in the human body, PAD1, PAD2, PAD3, PAD4 and PAD6 and they are distributed in a wide range of cell types and tissues (25).

1.1.2.2 Prevalence of ACPAs

ACPAs are only rarely found in other autoimmune diseases and in less than 2% of healthy individuals (26–28). Retrospective studies revealed that ACPAs can be present many years before onset of the disease (29). However, shortly before clinical onset of RA, ACPA titers increase and the ACPA profile broadens (30,31). Studies have shown that in 90% of patients with undifferentiated arthritis, such as joint pain or musculoskeletal symptoms, and ACPA positivity confers a risk of RA development suggesting the potential pathogenic role of ACPAs in RA (32,33).

1.1.2.3 Genetic and environmental Risk factors

Several genetic and environmental factors have been associated with ACPAs. The most prominent genetic predisposition is the so-called shared epitope (SE) in the human leukocyte antigen – certain DR isotype 1 (HLA-DRB1) variants e.g. HLA-DRB1*0401, *0404, *0101 and *1001.

The second strongest genetic predisposition is located in the protein tyrosine phosphatase non receptor type 22 gene (*PTPN22*). *PTPN22* encodes the protein lymphoid tyrosine phosphatase which negatively regulates T and B cell activation (34,35). An increase in a cytotoxic phenotype of T cells, Eomesodermin (EOMES+) CD4+ T cells with a more pronounced production of Perforin-1 has recently been described in *PTPN22* risk allele carriers (36).

1.1.2.4 Environmental risk factors for ACPA+RA

Environmental risk factors include air pollution such as smoking and inhalation of silica dust but also infectious agents (37,38). A strong interaction between the genetic risk factor HLA-SE alleles, smoking and ACPA positivity has been demonstrated, pointing towards a possible model for the aetiology of seropositive RA (38–40).

Recently, a connection between periodontitis and RA has been reported. Interestingly, the bacterium *porphyromonas (P.) gingivalis,* which is implicated in periodontitis, a chronic inflammatory oral disease, expresses a PAD enzyme and is therefore able to citrullinate proteins, which could suggest a model of molecular mimicry (41). This theory is further supported by the finding, that ACPAs are able to bind with citrullinated peptides from the bacterium *P.gingivalis* (42).

1.1.2.5 Characteristics of ACPAs

Numerous citrullinated proteins have been identified as targets for ACPAs, including filaggrin, fibrinogen, collagen type II, histones, alpha-enolase, tenascin-C and vimentin (43–47). Except for type II collagen, ACPAs most exclusively bind to the citrullinated form of these proteins. ACPAs have been identified as multi-reactive towards several citrullinated human proteins and peptides which appear to result from the recognition of ACPAs to specific motifs rather than specific proteins (48,49). Indeed, a recent study identified ACPAs which bound to a spectrum of modified proteins and extended the previously reported set of RA autoantigens (50).

ACPAs exhibit a high level of somatic mutations and distinct glycosylation pattern in the Fc and fragment antigen binding (Fab) region (48,51). Fc-glycan profiles in polyclonal ACPA-IgG exhibit a more pro-inflammatory profile with decreased terminal galactose and sialic acid residues, which is acquired prior to disease onset of RA (52–54). Fab-glycans, which is only accounted by 10-25% of IgG antibodies in the periphery and mainly originate from somatic mutations (55,56), are enriched in ACPAs and are highly sialylated which changes the charge and isoelectric point of the autoantibodies (57,58). Interestingly, antigen binding was not altered, which raises the question how and why Fab glycosylation is favored in ACPAs (57).

1.1.2.6 Effector function of ACPAs

In vitro studies demonstrated the ability of ACPAs to activate complement and interact with different cell types. ACPAs can stimulate macrophages through the Fc gamma receptor and Toll-like receptor 4 which leads to macrophage tumor necrosis factor (TNF-) production (59–61). Furthermore, ACPAs are able to induce NETosis of neutrophils, a regulated cell death of neutrophils in which neutrophils release a chromatin meshwork with antimicrobial peptides called neutrophil extracellular traps (NETs). NETosis is increased in blood and synovial fluid (SF) of RA patients and corelates with ACPA concentrations (62). Moreover, studies from our rheumatology division have also showed that ACPAs are capable of enhancing osteoclast maturation and bone destruction (17,49,63).

In experimental arthritis models, ACPAs alone are not capable to induce synovitis in mice, however, can significantly increase the severity of arthritis if injected with an inducer of arthritis (such as collagen II antibodies) (47,64). Even though ACPAs are incapable of inducing arthritis alone, intravenous injection of ACPAs reduced trabecular bone mineral density in mice. Moreover, injected mice exhibit a long-lasting pain like behavior in absence of inflammation. The pain behavior depended on the release of CXCL1/2 by osteoclasts which is bound to the receptor CXCR2 on sensory neurons (65). This finding could explain the arthralgia that develops before signs of joint inflammation and proceeds in RA patients even after treatment (66,67).

- ACPAs bind to specific motifs which are found on various proteins/peptides
- ACPAs are highly somatic hypermutated
- N-linked-gycosylation motifes are enriched the Fab region of ACPAs
- Fc-glycan profile of polyclonal ACPA-IgG changes prior disease onset
- Different binding patterns are associated with different fucntional properties of ACPAs

1.1.2.7 Citrulline reactive B cells in RA

ACPA positive B cells are enriched in the inflamed joint and circulate in the blood at a frequency of ~1 in 12,500 B cells (61,68,69). Additionally, ectopic germinal centers (GC) surrounded by ACPA positive plasma cells have been identified in synovial tissue of RA patients (70). ACPA expressing B cells exhibit signs of antigen-experience and are likely to be part of the chronic immune responses in RA (71). The majority ACPA positive B cells show a class switched memory B cells phenotype and substantial fraction is found in the circulating plasmablasts/plasma cells which resembles the dynamics of antigen-specific responses(72,73). Moreover, ACPA positive B cells display a high rate of somatic mutation and express genes that promote T-dependent B cell differentiation, class switching and nuclear-factor kappa B (NF-kappa B) signaling (15). In our unit, Joshua et al. demonstrated that ACPA reactive B cells are present in the lungs of seropositive individuals which are at risk for or having early untreated RA (74). Besides the lungs, we were also able to demonstrate the presence of ACPA positive B cells in inflamed gingival tissue (75).

These findings suggest that ACPA positive B cells are controlled by RA-specific CD4 T cell and repeated GC responses (71) and could originate from extraarticular locations such as the respiratory, oral or intestinal mucosa after infections or general immune activation caused by smoking or inhaling particles. Increased apoptosis and immune activation might stimulate citrullination which, in genetically predisposed individuals, might promote the generation of ACPAs. Over the years, ACPAs might undergo epitope spreading. However, it remains still unsolved how joint inflammation is initiated and how ACPAs contribute to the pathogenesis of arthritis (33,76,77).

1.2 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Systemic lupus erythematosus (SLE) is a heterogenic, systemic autoimmune disease involving a spectrum of clinical symptoms ranging from mild to life-threatening disease **(Figure 2).** It is characterized by remission and flares of disease activity and the clinical symptoms can differ from acute onset to chronic long-term disease (78,79).

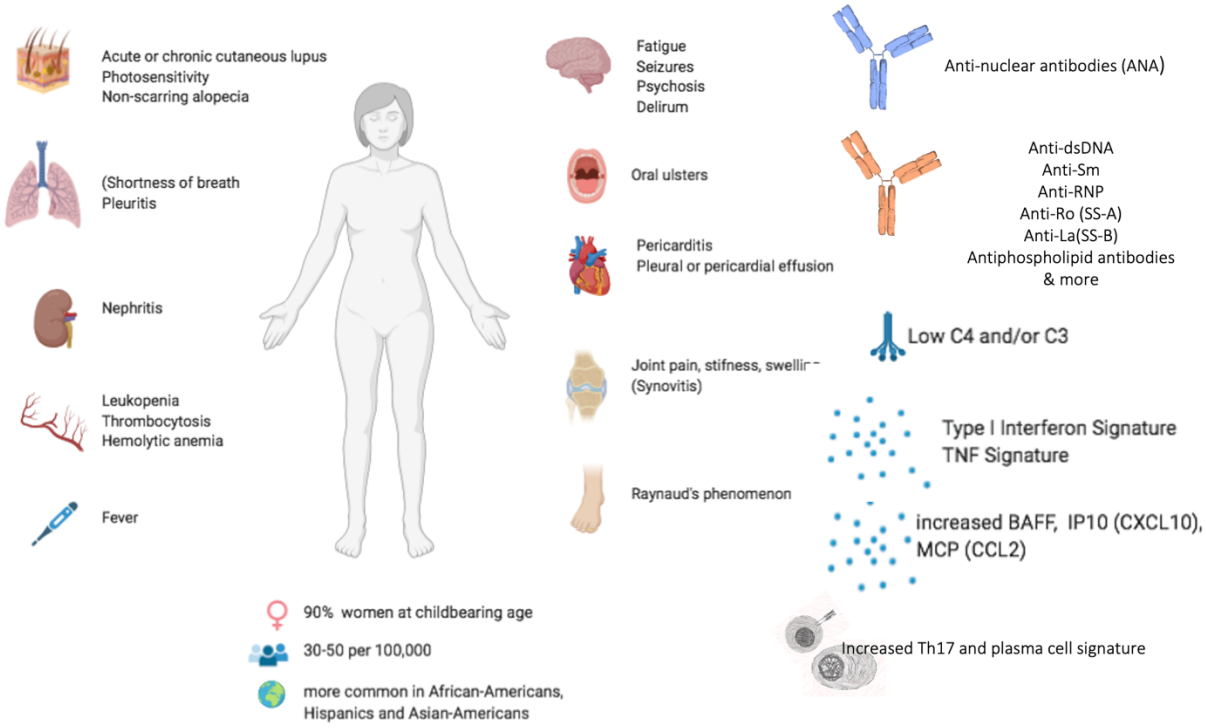


Figure 2: Characteristics of SLE patients. *Clinical manifestations of SLE patients according to the 2019 classification criteria. Prevalence of SLE is listed below the clinical characteristics and immunological features associated with SLE are shown on the right.* (3,80–82). *SLE is associated with the presence of a wide range of autoantibodies, proinflammatory cytokines and the consumption of complement. (created by Biorender).*

The majority (90%) of SLE patients are females, with disease onset during the childbearing ages, but SLE can manifest at any age (79). The survival rate of SLE patients improved dramatically over the last century. However, SLE is still associated with an increased risk for mortality compared to the general population (83,84). A recent study discovered that SLE is one of the leading causes of death in young women, ranking 10th among 15–24 year old females and 20th in females between the ages of 15-64 (85). Nowadays, one of the most common causes of death in SLE patients is cardiovascular disease which did not reduce over the last decades in comparison to other lupus activity related cause of death, such as renal disease (83,86).

Clinical manifestations include (87):

1. **Constitutional features** include fatigue (53-80%), fever (40-60%), lymphadenopathy and weight loss.
2. **Musculoskeletal involvement** affects over 80% of SLE patients, especially arthralgia (joint pain) which is often present without clinical evidence of inflammation.
3. **Cutaneous skin lesions** are common during the disease course, they can give rise to many different skin rashes, inclduing the butterfly (malar) rash, but skin manifestaitons can also excist as isolated disease (cutaneous lupus erythematosus) which is often linked to negative serology and less severe disease (88).
4. **Renal invovlment** occurs in 25-55% of SLE patients and is commonly associated with a more severe disease and reduced survival
5. **Haematological manifistations** consist of anemia (56%), thrombocytopenia, leucopenia, lymphopenia (76%) and clotting abnormalitities.
6. **Neuropsychiatric disease** include a range of neurological and psychiatric syndrome observed in SLE including depression, psychosis (2%), siezures, demyelination (<5%), neuropathy and cognitive dysfunction
7. **Cardiorespiratory problems** such as pleural disease (45-60%) and pericarditis (50%)

Even though women are predominantly affected, men often display a more severe disease with regards to both renal and extrarenal manifestations (89).The incidence and prevalence of SLE varies between studies, countries and ethnicities. SLE is more common in African Americans, Hispanics and Asian Americans who often also show a more severe disease course (90). Data from national Swedish registers have shown a prevalence ranging from 46-85 cases per 100,000 in Sweden. Recent data also demonstrated geographic differences and varying female to male ratios (90).

SLE is associated with the presence of a broad spectrum of autoantibodies, which is a sign for the break of immune tolerance. Certain autoantibodies are stable over the disease course while others fluctuate and are associated with disease activity (87). Over 95% of SLE patients with active disease are positive for anti-nuclear antibodies (ANA) and thus, they are an important biomarker for the disease. Indeed, ANA positivity at least at one timepoint is the obligatory entry criteria in the new classification criteria of SLE. This is followed by seven clinical manifestations and three laboratory factors (89). The first classification criteria for SLE were published 1971 and revised in 1982 (91). In 2019 the new criteria were published (89).

Interestingly, autoantibodies can be detected many years before the onset of clinical symptoms in the majority of patients (90%). However, the appearance of new types of autoantibodies increases up to the disease diagnosis, suggesting that the autoimmune processes already start many years before diagnosis (92).

Thus, SLE pathogenicity can be divided into three main stages, which will be discussed in detail in the following sections.

1. Genetic susceptibility and environmental factors
2. Break and loss of tolerance to self-antigen
3. Inflammation, tissue damage, clinical manifestations

1.2.1 Genetic predispositions in SLE

Genetic predispositions play an essential role in SLE pathogenesis which is evident from the heritability (44%) and relative risk (6%) in first-degree relatives o SLE patients (93). Over 100 genetic loci have been associated with SLE susceptibility, are often shared across ethnicities and with other autoimmune diseases (94).Interestingly, family studies reported an increased risk of other autoimmune diseases in the same family with SLE individuals (93). Indeed, 44% of the immune-mediated risk single nucleotide polymorphism (SNPs) are associated to multiple autoimmune diseases (95).

In most cases, SLE is influenced by a combination of common polymorphisms. Common genetic risk factors affect proteins from following pathways (94):

- Complement pathway (C1Q,R;S, C2, C3, C4A&B)
- NF-kappa B signaling (TNFA/P3, PRKCB, TNIPI)
- TLR and interferon I signaling (IRF5, IRF4, TLR7,8,9)
- DNA degradation, apoptosis and clearance of cellular debris (RAD51B, TREX1,DNASE1, ATG5)
- Immune complex processing and phagocytosis (FCGR2A,ITGAM)
- B-cell and T-cell function and signaling (PTPN22, TNFSF4, CD44, LYN, BLK, HLA-DR2/DR3; DRB1)
- Cytokines (IL-10, IL21, IL-6)

One of the most impotent risk factors include the *HLA* region(HLA-DR2 (DRB1*15:01), HLA-DR3 (DRB1*03:01), HLA-DRB1*08:01, and HLA-DQA1*01:02 alleles), *STAT4* and interferon I related genes such as I*RF5* (96).

SNPs in the *PD-1* gene, a regulator for B and T cells, exhibited a strong correlation as well (94). It is noteworthy, that a single mutation is sometimes enough to develop disease. These single mutation are within the genes encoding the complement component 1q (C1q) subcomponent A (C1QA), C1QB, C1QC, three-prime repair exonuclease 1 (TREX1), or deoxyribonuclease 1-like 3 (DNASE1L3) (97). Moreover, certain SNPs also confers a risk for specific clinical manifestations of SLE. The Integrin Subunit Alpha M (ITGAM/CD11b) risk allele was associated with an increased risk of renal disorder and discoid rash (98).*TNFSF4* risk allele was associated with renal involvement in SLE patients. The expression of TNFRSF4 on CD4+ T cells is associated with nephritis and disease activity (98,99). SLE patients with leukopenia exhibited a higher presence of the IL-21 risk allele (98).

In addition to genetic variants, epigenetic changes trough alteration of DNA methylation may exert the influence of genetic risk variance (89).

1.2.2 Environmental factors in SLE

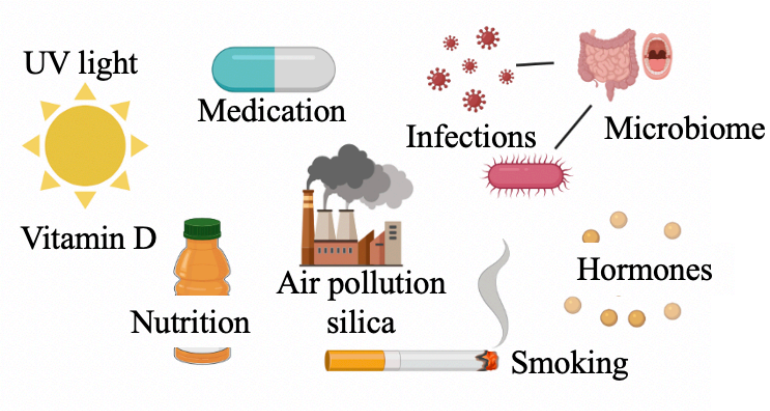


Figure 3: *Possible environmental risk factors of SLE include ultraviolet (UV) light exposure, Vitamin D deficiency, air pollution, silica dust smoking, medication, hormones and infections (101) (created by Biorender).*

Exposure to strong silica dust, ultraviolet light, smoking, air pollution, vitamin D deficiency, nutrients, solvents and viral and bacterial infections such as EBV have been suggested as environmental factors for SLE (**Figure 3** (100)).

Hormones might concur to the occurrence of SLE given the fact that SLE is more common in young women post-puberty (100). Oral contraceptives. and postmenopausal hormone replacement therapies have been linked with an increased risk for SLE incidence (101). Bernier et al. reported a strong-dose relationship between new onset SLE and the oral contraceptive ethinyl estradiol (102). Interestingly, low levels of dehydroepiandrosterone (DHEA), an endogenous steroid hormone precursor, have been linked with predisposition to SLE, and clinical studies suggested that treatment with DHEA in mild to moderate disease might be beneficial, however DHEA substitution only showed modest effects in the clinic (103,104).

Certain drugs (f.e. Procainamide, Hydralzine) are also able to provoke SLE-like clinical features and are associated with anti-histone antibodies. Drug-induced lupus is usually less severe and resolves in most cases after discontinuation of the offending agent (105).

The microbiome have gained more and more attention over the last couple of years and several research groups investigated the link between SLE and commensal bacteria (106). In general, SLE patients exhibit a less diverse gut microbiota compared to the general population (106). SLE patients are suggested to have an impaired gut barrier.
A study by Manfredo et al. demonstrated that the commensal bacterium, *Enterococcus gallinarum*, translocate from the small intestine to the liver in some SLE patients and in autoimmune-prone mice (107). In mice, this translocation resulted in a systemic type I interferon signature and anti-dsDNA production(106). Furthermore, Greiling et al. discovered that the human Ro60 epitopes were highly homologous with commensal bacterial Ro60 orthologues (108). Anti-Ro60 antibodies from SLE patients were able to cross-react with the bacterial version of Ro60 indicating that pathological autoimmunity could arise from a pathobiont (an organism that can cause harm in certain situations but otherwise lives as symbiont) in predisposed individuals (108,109).

1.2.3 Autoantibodies in SLE

A wide spectrum of autoantibodies can be detected in SLE patients. Certain autoantibodies have been associated with specific clinical features and are linked to particular genetic risk factors. This chapter will discuss specific autoantibodies found in SLE (**Table 1**).

Table 1: *Characteristics of Autoantibodies in SLE (adapted from* (87) *with additional data from* (110–112)

Autoantibody	Prevalence	Clinical relevance	Other diseases
ANA	95%	Entry criteria in titer 1/80 for SLE, according to the EULAR-ACR classification criteria	Mixed connective tissue disease, scleroderma, SS RA, polymyositis, dermatomyositis, drug-induced
anti-dsDNA	40-98%	Often linked to disease activity And lupus nephritis	Rare in other diseases
anti-nucleosome	85%	Lupus nephritis	Autoimmune hepatitis, mixed connective tissue disease, scleroderma, SS
anti–SSA/Ro	25-40%	Sicca symptoms, subacute cutaneous SLE, pneumonitis, shrinking lung syndrome, lymphopenia, nephritis, vasculitis, thrombocytopenic purpura, ocular damage, congenital heart block, skin rash in newborn	SS, RA, polymyositis, scleroderma, subacute cutaneous lupus
anti–SSB/La	10-22%	Congenital heart block	SS
anti-Smith	20-30%	Mortality, serositis, nephritis, neuropsychiatric SLE, pulmonary fibrosis, leucopenia, arthritis, malar rash, discoid rash, vasculitis, pulmonary hypertension, peripheral neuropathy	Rare in other diseases
anti-snRNP/U1-RNP	25%	Interstitial lung disease, neuropsychiatric lupus, fevers, myositis, pleuritis, Raynaud's phenomenon, leukopenia	SS, RA, polymyositis, scleroderma
anti-ribosomal P	10-20%	Hepatitis, neuropsychiatric SLE	Hepatitis
anti-phospholipid	30-40%	Thrombotic events, pregnancy complications and loss, Thrombocytopenia	Infections, malignancies, primary APS
anti- C1q	33%	Nephritis	SS, RA, vasculitis
anti-histone	70-80%	Drug-induced	RA, SS, JIA, vasculitis
RF	12.9-20%	Sicca syndrome, hypergammaglobulinemia, anemia, leukopenia	RA, infections
anti-CCP	5.5%	Erosive or deforming arthritis	RA

Abbreviations: ANA: anti-nuclear antibodies, SS: Sjogren syndrome RA: rheumatoid arthritis, APS: antiphospholipid syndrome, C1q: anti-complement component 1q, RF: rheumatoid factor, CCP: cyclic citrullinated peptide; snRNA: small nuclear ribonucleoproteins, anti-SSA/SSB: Anti–Sjogren's-syndrome-related antigen A/B autoantibodies

1.2.3.1 Anti-nuclear antibodies (ANA)

ANA comprise a heterogenic group of autoantibodies targeting antigens in the cell nucleus and are divided in two main groups(113):

1. DNA and Histones
2. Extractable nuclear antigens

ANA can be present in several autoimmune diseases, cancers, drug-induced or infectious diseases as well as in of healthy individuals (113,114). They are detected by immunofluorescence antinuclear antibody test or enzyme linked immunosorbent assay (ELISA). For the immunofluorescence antinuclear antibody test, human laryngeal carcinoma cells (Hep2 cells) are incubated with patient serum. The bound autoantibodies are then captured by fluorescent-conjugated detection antibodies and visualized by fluorescence microscopy (115,116). The resulting staining patterns identify different ANA subtypes and can be used to diagnose different diseases. A homogenous nuclear pattern is associated with anti-DNA, anti-histone and nucleosome antibodies and common in SLE (especially drug induced SLE), RA and juvenile chronic arthritis (115). Fine, tiny speckles indicate autoantibodies binding to nuclear proteins such as RNP, Smith, SSA/Ro, SSB/La, Scl-70, Jo-1, ribosomal-P and are linked to SLE, myositis, and SS (113,115).
The detection of different ANA subtypes in this assay, makes it a valuable diagnostic tool.
A positive ANA test is nowadays an entry requirement in the new classification criteria of SLE (3). However, ANA antibodies can also be frequently detected in a healthy individual especially after infections and other autoimmune diseases. Thus, a positive ANA test alone, without clinical symptoms, has no diagnostic value (117).

1.2.3.2 Anti-DNA antibodies

As mentioned above, anti-DNA antibodies are a subtype of ANA antibodies and they can either bind single- or double-stranded DNA. Anti-dsDNA antibodies can be detected in 50-70% of SLE patients, correlate with disease activity in the majority of patients and are rare in other autoimmune diseases (87,118). Most, but not all, anti-DNA antibodies have been shown to be somatically mutated, suggesting that they originate from germinal center reactions (119).

1.2.3.3 Anti-SSA/Ro and anti-SSB/La antibodies

Anti–Sjögren's-syndrome-related antigen B autoantibodies (Anti-SSB/La) are almost always found together with anti–Sjögren's-syndrome-related antigen A autoantibodies (anti-SSA/Ro) while anti-SSA/Ro can also be present independently. Both autoantibodies are able to cross the placenta resulting in neonatal lupus and atrioventricular heart block in the child (87).

1.2.3.4 Anti-phospholipid antibodies

Antiphospholipid antibodies are heterogenous group of autoantibodies composing of autoantibodies which bind to:

1. anionic phospholipids (i.e. cardiolipin, phosphatidylserine)
2. phospholipid binding proteins (i.e. β 2 Glycoprotein I (β2GPI), Annexin V, prothrombin)
3. phospholipid – phospholipid binding protein complexes

The persistent presence of antiphospholipid antibodies is associated with recurrent venous arterial and small vessel thromboses and/or pregnancy complications such as spontaneous miscarriages, preeclampsia and less frequently maternal thrombosis (120). Antiphospholipid antibodies are found in around 30-40% of SLE patients from which 20 to 50% develop thrombotic complications (121). In SLE patients, antiphospholipid antibodies are associated HLA-DRB1*04 and HLA-DRB1*13 alleles (122).

Three autoantibodies are currently part of the classification criteria of antiphospholipid syndrome (APS) and SLE (3): lupus anticoagulant, anti-cardiolipin and anti-β2GPI. LA assay measures the clotting time in patients, which is prolonged in APS patients. Since it is a functional test, it detects the presence of several different antiphospholipid antibodies inducing antibodies against prothrombin, β_2GPI but also against protein C/S, Annexin V and protein-protein complexes such as β_2GPI – cardiolipin or phosphatidylserine – prothrombin. The presence of those antibodies is believed to prolong the activation of the coagulation cascade by interfering with its phospholipid-dependent steps (120,123–125).

1.2.3.5 Rheumatoid Factor (RF) in SLE

RF can be detected in SLE patients with different frequencies depending on the isotype: IgM 17.9%, IgG 20.5% and IgA 20.5%. Witte et al. found an association between IgA RF and HLA-DR3, high concentration of ANA antibodies, as well as anti-SSA/Ro and SSB/La antibodies (92).

1.2.3.6 Anti-citrulline-reactive autoantibodies (ACPA) in SLE

ACPAs are present in a subgroup of SLE patients and have been associated with erosive arthritis and deformations (126–128). However, ACPAs can exists in lupus arthritis patients without erosion(129–131). Interestingly, CCP positivity and erosion were associated with HLA-DRB1*0401 and HLA-DQB1*0302 shared epitopes alleles in SLE patients(132). In addition, high CCP titers have been linked to a small subgroup of patients (Rhupus) which features RA and SLE characteristics simultaneously in the same patient (125,128).

1.2.4 Impaired clearance of apoptotic debris in SLE

In some SLE patients, an impaired clearance of apoptotic bodies has been described and suggested to be responsible for the break of tolerance. Macrophages from SLE patients exhibit a reduced phagocytic activity (134,135) and an accumulation of apoptotic debris in tissue, such a skin and bone marrow (136,137). Moreover, it has been demonstrated, that NET formation, a specific cell death in neutrophils, is increased and insufficiently cleared in SLE patients(138). Bauman et al. investigated lymph node biopsies from SLE patients and found that a subgroup of patients presented a reduced number of tingible body macrophages, a specific type of macrophages which are mainly present in germinal centers (GCs) (139,140). This was linked to a reduced clearance of apoptotic cells and an accumulation of apoptotic bodies in the GCs (141). The lack of tingible body macrophages in addition to the presence of apoptotic bodies could serve as a survival signal for autoreactive B cells binding self-antigen and especially nuclear content (142). However, the impaired clearance could have also originated from the disease itself, e.g. by autoantibodies inhibiting the clearance of apoptotic bodies (141)

1.2.5 Type I interferon (IFN) signature in SLE

SLE patients display increased levels of type I Interferon as well as elevated expression of interferon related genes in blood and tissue (138,143,144). IFN-α activity is higher in younger patients and IFN-α levels correlate in some studies with disease activity, but in general show a rather stable pattern over time (81,145,146). Type I IFN are normally involved in the host defense mechanism against viral infections. In peripheral tissue of SLE patients, infiltrating plasmacytoid dendritic cells (pDCs) represent the main source of type I IFN (147). Other cells such as monocytes and neutrophils have also the capacity to produce type I IFN. Interestingly, cancer patients treated with IFN-α can develop a lupus-like syndrome with the presence of anti-nuclear autoantibodies which suggests that high levels of type I IFN could be able to break down tolerance mechanisms and provoke autoimmunity (81,148) Type I IFN production can be induced by immune complexes in particular in combination with necrotic

or apoptotic cell content (149,150). IFN can then act either directly or indirectly on B cells, by inducing the B-cell activation factor (BAFF), reducing the BCR activation threshold and warrants naïve B cells to respond against TLR7 ligands(143,151,152). Furthermore, IFN production in bone marrow of SLE patients by neutrophils has been linked to a reduction in frequency of early B cell subsets (153).

Besides type I IFN, IFN type II and III have also been associated with SLE and correlated with disease activity(154,155). Interestingly, IFN-γ levels, a type II IFN, are increased years before the diagnosis of SLE while in the more proximate pre-disease phase, elevated levels of type I IFN are detected (153). Recently, Oke et al. showed that high levels of different IFNs are associated with distinct clinical features (155). On the one hand, high IFN-γ and high type I IFN scores, measured by upregulated IFN genes in cell cultures after exposure to SLE serum, correlated with nephritis and arthritis. On the other hand, high IFN-α levels were linked with active mucocutaneous inflammation and a more benign cardiovascular profile and isolated IFN-λ1 levels were associated with less severe disease (155).

1.2.6 B cell abnormalities, disfunction and hyperreactivity

B cells are important player in the pathogenicity of SLE and contribute to the disease in numerous ways (88). B cells secrete autoantibodies, process and present antigens to T cells and produce anti- and proinflammatory cytokines (156). In addition, several genetic risk alleles are associated with B cells signaling and can lead to B cell hyperresponsiveness in SLE (82).

During B cell development in the bone marrow (BM), early B cells undergo clonal deletion and receptor editing (157). This process is regulated by central tolerance checkpoints. In healthy individuals, this checkpoint eliminates around 20-50% of autoreactive B cell clones (158). A second checkpoint occurs in the periphery during the transition to mature B cells. Self-reactive clones are eliminated or silenced by anergy. Break of tolerance has been described in SLE (159). SLE patients show defects in several tolerance checkpoints resulting in the accumulation of self-reactive and polyclonal B cells in the periphery regardless of the disease status (160–162). Overall, SLE patients exhibit a higher number of self-reactive and polyreactive naïve B cells suggesting that more autoreactive B cells might enter the germinal centers (GC). GCs are the primary site of clonal B cell expansion and affinity maturation. Thus, they play also an essential role in autoreactive B cell development and repertoire. B cells in the GC circle between dark zone and the light zone. In the dark zone, B cells divide

and obtain somatic hypermutation whereas they undergo selection of high affinity antigen BCRs in the light zone (157,163). B cell entry into the GC and selection of B cells is highly regulated by T cells, dendritic cells, macrophages and stromal cells (157). In SLE, GC tolerance mechanisms seem to be subverted. In healthy individuals, naïve- autoreactive clones are excluded in early-GC stages while they contribute to GC reactions in SLE patients resulting in an expansion of autoreactive post-GC B cell phenotypes (164).

Specific autoantibodies are frequently mutated, and ectopic GC can be found in the kidney of nephritis patients (165,166). It is likely that autoantibodies can be generated from non-autoreactive, polyclonal and autoreactive precursors (166,167). Mietzner et al. analyzed autoreactive IgG antibodies and demonstrated that these autoreactive clones arise from non-autoreactive or polyreactive precursors, which supports the idea that somatic mutations contribute to the autoantibody specificity and reactivity (166).

Peripheral tolerance can be challenged by extrinsic and/or intrinsic factors (168):

Intrinsic Factors	Extrinsic Factors
• BCR signaling • TCR responses	• Incomplete clearance of apoptotic cells • Hyperactive T helper cells and dendritic cells • Cytokines (BAFF, TLR stimulation)

Recently, Gies et al reported two dysregulated features of B cells in SLE patients: reduced expression of the CD19/CD21 complex already in transitional B cells and defective TLR9 functions in naïve B cells from untreated SLE patients (169). TLR9 is important in governing B cell responses to DNA-containing antigens in the periphery by inducing apoptosis (169,170). Additionally, TACI, a specific BAFF receptor, plays a crucial role during B cell activation and removal of autoreactive B cells (159). TLR7 also has been shown to be instrumental in the pathogenic mechanism of SLE. It is essential for the generation of spontaneous GC as well as GC and extrafollicular responses (171,172).

Furthermore, aberrations of specific B cell compartments **(Figure 4)** have been associated with SLE and were found to correlate with clinical features including disease activity and antibody titers (173).

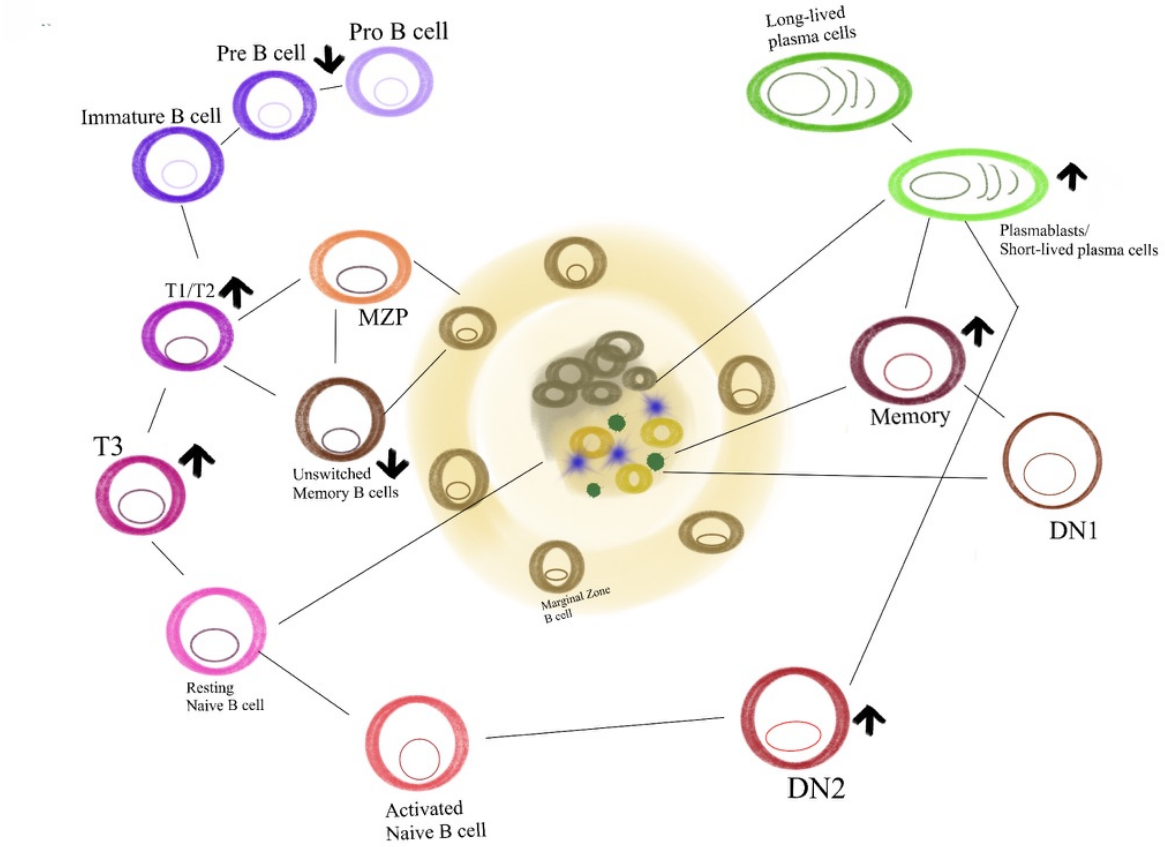


Figure 4: *B cell fate starting from the development in the bone marrow to long lived plasma cells. Arrows indicating an increase or decrease of the subset in SLE. Adapted from:* (174)

1.2.6.1 Pro/pre and Transitional B cells

In BM of SLE patients, pro/pre B cells are reduced and an expansion in the transitional B cell subset has been reported (153). This phenomenon was linked to an increased IFN-signature and high BAFF/APRIL levels in BM (153). The frequency of transitional B cells (CD19+CD7 $CD24^{hi}CD38^{hi}$) is increased in SLE patients compared to RA and healthy individuals (175,176). Interestingly, transitional B cells are able to produce IL-6. Furthermore, IFN-alpha has been shown to enhance the survival of transitional B cells (177).

1.2.6.2 Unswitched and switched memory B cells

Memory B cells (IgD-CD27+) are increased in SLE patients and have been shown to be less susceptible to immunosuppressive therapy (178). Memory B cells have the ability to quickly differentiate into antibody-secreting cells, or alternatively, enter the germinal center upon antigen stimulation, which accelerates the secondary immune responds towards previously encountered pathogens (179).

In SLE, switched memory B cells are increased particularly in LN patients in comparison to non-LN and healthy individuals (177). The high frequency might result from the persistent

antigen stimulation, chronic inflammation and presence of spontaneous germinal centers in the kidney. In addition, Zhu et al. demonstrated a decrease of switched memory B cells in new onset SLE patients in comparison to healthy controls and chronic SLE patients (177), which could have resulted from an activation and differentiation into antibody secreting cells and highlights their potential pathogenic role in the diseases.

Interestingly, non-switched memory B cells (IgD+CD27+) are permanently decreased in SLE patients (180). Their origin and function remains unclear even though they might correspond to circulating splenic marginal zone B cells (180).

1.2.6.3 Antibody secreting cells/ Plasma cells

After antigen stimulation, B cells can develop into short- and long-lived plasma cells .While short-lived PC primary accumulate in tissue, long-lived PC migrate to the bone marrow into specific niches where they can survive for many years and are responsible for persistent antigen-specific antibody titers (181). Plasmablasts are precursors of circulating plasma cells in the blood which will reach either the bone marrow, spleen or lymphoid tissue and can be either long or short lived (156).

Plasmablasts are elevated in peripheral blood of active SLE patients and are associated with increased autoantibody production (173,182,183). A recent study by Tipton et al demonstrated that active SLE patients display an increase in newly generated antibody secreting cells, with low somatic hypermutations and an expansion of autoreactive VH4-34 cells. A substantial fraction of these antibody secreting cell was derived from newly activated naive B cells, which display a similar phenotype to double negative 2 (DN2) B cells (which is explained in the next section) (184,185).

Interestingly, different autoantibodies show different pattern of expression, some which correlate with disease activity and fluctuate (anti-dsDNA) and some which have a stable expression over time and are not as affected by immune suppression (anti-RNP, anti-SSA/SSB). This phenomenon might suggest that more pathogenic autoantibodies are produced by short lived plasma cells and plasmablasts which might evolve from a new wave of activation of naive or memory autoreactive B cells (118).

A recent study by Mei et al. discovered that a proportion of plasmablasts in peripheral blood of SLE patients which exhibited a mucosal phenotype similar to antigen-specific plasmablasts which can be found after oral vaccination (177). This suggests that a proportion of plasmablasts in SLE patients might originate from mucosal sides of the body.

1.2.6.4 Double Negative (DN) B cells

Expansion of the IgD-CD27- (DN) B cell population is associated with clinical manifestations and severity of SLE (186,187). The DN B cell population consists of a heterogenic group of B cells including the so called 'atypical memory B cells', DN2 cells or age-associated B cells (ABCs). Different research groups have identified similar B cell subsets enriched in SLE patients using different surface and intracellular markers:

Fleischer et al. described an enriched B memory subset in SLE patients, expressing high levels of spleen tyrosine kinase (Syk), high levels of CD19 and CD20 but low levels of CD27 and CD38 and exhibited enhance differentiation into antibody-secreting cells. Jacobi et al. discovered 'activated memory B cells' in the DN subset using the marker CD95. These B cells also expressed low levels of CD21 and CXCR5. Interestingly, DN CD95+ B cells were primarily isotype switched and showed similar mutation patterns to typical IgD- CD27+ B cells (181). Jenks et al. described an enrichment of DN B cells in SLE patients which is primarily caused by the CD11c+CXCR5- (DN2) subset (188).

Later studies revealed that these B cells represent precursors of antibody forming cells which are enriched in the autoreactive BCR repertoire (189–191). This novel B cell population correlated with clinical outcomes and disease activity in SLE (192). The exact phenotype and the challenges of identifying these cells are discussed in detail in the next section.

1.2.6.5 Age associated B cells, T-bet expressing B cells, exhausted B cells, DN2, "activated memory" - How should we call them?

A B cell subset expressing T-bet was first described in 2000 and ascribed to IFN-γ production (193). The first indication of the pathogenic role of T-bet expressing B cells was reported by Peng et al. in 2002. In this report, T-bet-deficient B cells demonstrated an impaired production of IgG class switching (IgG2a, IgG2b, and IgG3) and the absence of T-bet in B cells in lupus prone mice reduced autoantibody production, and immune-complex driven renal disease (194). Next, an enrichment of CD21 negative B cells has been revealed in several autoimmune diseases (195,196). Isnardi et al. analyzed CD21 negative B cells in RA and common variable immunodeficiencies (CVID) patients and found germline autoreactive antibodies, which recognized nuclear and cytoplasmic structures as well as unresponsiveness towards BCR or CD40 stimulation(196). In 2008, CD11c+ "plasmablasts" were described in the spleen of mice after a bacterial infection with *Ehrlichia muris* (193) and an expansion of "exhausted memory B cells" with the phenotype $CD21^{low}$CD27- in HIV patients (193). In 2011, two independent groups discovered a novel B cell subset, expressing CD11c and lacking the expression of CD21 and CD23. The subset was led by the transcription factor T-

bet and accumulated with age (188,197), and thus were called age-associated B cells (ABCs). Besides their correlation with age, an increase in ABCs was soon found in several autoimmune diseases (SS, RA, SLE, MS) as well as after various infectious diseases (malaria and HIV) (198,199).

This ´atypical B cell subset´ does exist in healthy individuals. CD21 negative B cells exist in healthy individuals and account for approximability 5% of human B cells. The CD21- subsets were further divided in CD38-CD24+ (mainly CD27+ IgD+ IgM+) and CD38-CD24low cells (switched, CD27-). Stimulation of TRL-7/8 and IL-21 induced differentiation into antibody-secreting cells (193). CD21 is a BCR co-receptor binding fragments of the complement C3 and thereby lowers the threshold for antigen stimulation(193). CD11c+ B cells were also investigated in healthy humans and the analysis by Golinski et al. revealed that CD11c+ B cells are a heterogenic population which primarily exhibit the phenotype of memory B cells and overexpress genes involved in B cell activation, differentiation and antigen presentation (194).

In SLE, Wang et al. described an expanded population of $CD11c^{high}$ T-bet+ cells and performed an in-depth analysis of $CD11c^{high}$ T-bet+ cells (200). This population was enriched in autoreactive clones and correlated with clinical features. In response to IL-21, $CD11c^{high}$ T-bet+ cells were demonstrated to differentiate into Ig-secreting autoreactive plasma cells. Moreover, $CD11c^{high}$ T-bet+ cells did not correlate with age (200).
Jenks et al. explored the expanded double negative (CD27- IgD-) subset in SLE patients using the markers CD11c, CXCR5 and divided the DN population into DN1 (CXCR5+CD11c-) and DN2 (CXCR5-CD11c+) cell subsets (188). While the DN population in healthy individuals primarily accounted for DN1 cells, DN B cells in SLE had an increased population of DN2 cells. DN2 cells express low levels of CD21, CD24, CD38, and CD62L but have a higher expression in HLA-DR, CD86 and CD69 indicating a resemblance of DN2 to ABCs (188). Similar to $CD11^{high}$ T-bet+ cells, DN2 cells rapidly differentiated into antibody secreting cells following IL-21 and TLR7 stimulation (188). The importance of TLR7 signaling for the development of ABCs has previously been described (189). Furthermore, DN2 frequency correlated with serum type I interferon activity and higher anti-RNA antibody titers, anti-Smith and anti-RNP antibodies (188).

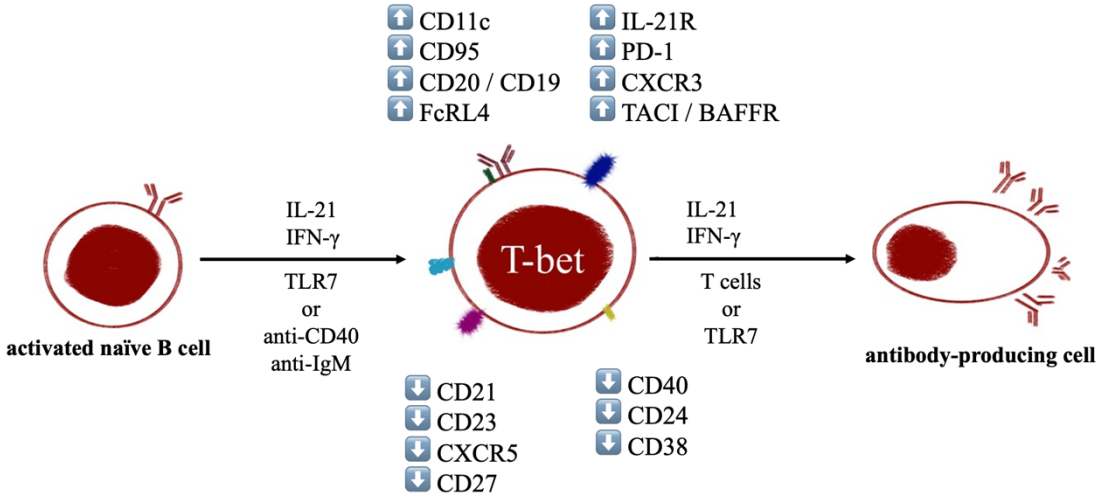


Figure 5: Characteristics of double negative 2 (DN2) / age/autoimmune-associated B cells (ABCs). *The ABC- phenotype can be induced by IL-21, IFN-γ and TLR7 or anti-CD40 and anti-IgM. ABCs are described by the up and downregulation of specific expression markers as well as the transcription factor T-bet. In response to IL-21, IFN-γ and TLR7 stimulation or T cells, ABCs have been shown to quickly differentiate into antibody producing cells.*

In conclusion (**Figure 5**), this ‘atypical` B cell subset has been shown to express CD11c, CD20, CD95,FcRL5 and CD86, the transcription factor T-bet and expresses low levels of CD21, CD23, CD38, CD40 and CD27 (188,189,197,200). Furthermore, two BAFF receptors were found on this B cell subset (200). They are enriched in autoimmunity and after antigenic stimulation. They are plasma cell precursors due to their ability to quickly differentiate into antibody secreting cells through the stimulation of TLRs and cytokines. Furthermore, ABCs have been demonstrated to contain autoreactive B cells and are likely to contribute to the pathogenicity of autoimmune diseases (199).

Up to this moment, different research groups use different identification marker and names for this unique B cell subset. This provides several draw backs and challenges. Using only one single marker, can result in an extremely heterogenic population of B cells. Furthermore, it is difficult to compare and find relevant studies if different markers and names are used to identify this subset. Thus, I believe it is of great importance to come to a common consensus about what markers are best to include in identifying this unique B cell subset by comparing RNA seq and flow cytometry data. In this thesis, this unique B cell subset will be referred to as ABCs, short for autoimmune-/age-associated B cells, if the markers CD11c, CD21 were used and DN2 if the markers CD11c and CXCR5 were used.

1.2.7 T cell involvement in SLE

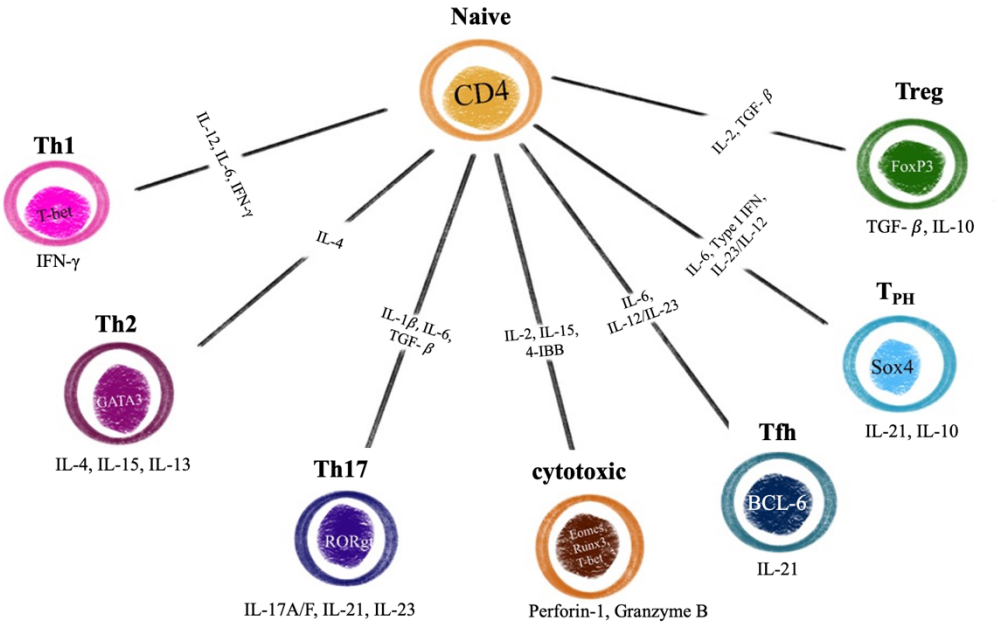


***Figure 6**: **T-helper cell phenotypes.** Activated naive CD4+ T cells can differentiate into various T-helper cell subsets which fulfil different effector functions.*

T cells and T:B cell interaction are essential players in SLE. Several genetic predispositions might lead to abnormal T-cell signaling and/or interactions in SLE patients, such as antigen-presentation to T cells through the MHC molecules. T cells are key players in regulating immune responses by driving inflammatory and regulatory processes as well as antibody production. In SLE patients, T cells have been shown to exhibit a number of biochemical and functional abnormalities (201). T cell transcriptome analysis by Bradley et al. demonstrated that T cells from SLE patients show alterations in several pathways related to mitochondria, metabolism, DNA replication and cluster in different patient subgroups (202), which highlights the heterogeneity of the disease.

Naïve CD4+ T helper (Th) cells can differentiate into various effector/memory and regulatory T-cell subsets which are indicated in **Figure 6.**
In SLE patients, Th cells are skewed towards the Th17 phenotype with downregulation of Th1 and T regulatory (Tregs) cells (203). Th17 cells are able to produce the cytokines IL-17, IL-21 and IL-23 which fulfill several different functions.

Function of IL-17, IL-21 and IL-23

IL-23 promotes Th17-cell mediated tissue inflammation as well as Tfh and DN T cell expansion (376,377).

IL-21 has broad range of function and acts on several different cell types. Its main source of production are T follicular helper cells but is also produced by neutral killer T cells, CD8 T cells and Th17 cells (179). IL-21 has shown to induce differentiation of naïve B cells into ABCs and ABCs into antibody-secreting cells (185,200).

The **IL-17 family** consist of six members: A, B, C, D and F. IL-17A is the most studied cytokine from this family and IL-17F is most commonly produced by IL-17A producing cells. IL-17A has been shown to induce production of inflammatory cytokines and promotes recruitment of inflammatory cells such as monocytes and neutrophils at the site of inflammation (348,378). IL-17A can be produced by various T cells including Th17 cells, γδ T cells, double negative (DN) T cells and CD8+ T cells as well as by macrophages, mast cells and neutrophils (377). The functions and roles of the other members of the IL-17 family are still poorly understood (43).

Th17 and the cytokine IL-17 are enriched in peripheral blood of SLE patients and in kidney biopsies and contribute to tissue inflammation and organ damage (80,201).

Tregs, play an important role in suppressing and regulating the immune system and are defined by the expression of CD25 and the transcription factor FOXP3 and can either develop from the thymus or at the side of inflammation (179). In SLE, Tregs are especially decreased in peripheral blood of patients with active disease (204,205). Tregs and Th17 cells are interlinked with each other. Th17 cell polarization is induced by STAT3 and the transcription factor retinoid acid related-orphan nuclear receptor γt (RORγt). This polarization can be shifted towards T regs via STAT6 and the induction of FOXP3, which is a negative regulator of RORγt (206) and an important tolerance mechanism. The Th17/Treg balance can be altered by proinflammatory cytokines, such as IL-1β, TGF- β and IL-6 which inhibit the FOXP3 (80) and favor the differentiation into Th17 cells.

Th17 cells are a plastic and heterogenic T cell subset, and their function depends on the local cytokine milieu. Stimulation with IFNα/β results in IL-10 production while IL-23 reduces the production of IL-10 and induces IL-17 production (80). Furthermore, Th17 cells are able to transdifferentiate and adapt functions from other T cell subsets, such as IFN-γ producing Th1 like cells or Tfh-like cells in Peyer's patches to support B cells (205,207).
Aberrant T cell signaling has been described in SLE and could contribute to the skewing of T cells towards IL-17 producing cells (201). Protein phosphatase 2Ac is increased in T cells of SLE patients and promotes glomerulonephritis in transgenic mice by increased production of IL-17A and IL-17F (208). Dysfunction of Rho-associated protein kinase (ROCK) and its pathways has been reported in SLE patients, which leads to increased T cell polarization, adhesion and migration (209). In mice, ROCK2 facilitated the activation of IRF4, which is obligatory for the production of IL-17 and IL-21 (210). In addition, cAMP response element modulator, calcium/calmodulin-dependent protein kinase IV are increased in SLE patients and contribute to IL-17 production and differentiation of Th17 and double negative T cells (201,211,212).

Double-negative T cells (DN, CD4−CD8-), express the αβ TCR but lack the expression of the coreceptors CD4 and CD8. In healthy individuals, DN T cells are suggested to exert strong immunosuppressive roles (213). DN T cells are enriched in peripheral blood of SLE patients especially in lupus nephritis patients and infiltrate the kidney (214,215). However, in contrast to healthy individuals, DN T cells from SLE patients have been identified to produce IL-17A and IFNγ and if stimulated in vitro, undergo a vigorous proliferative responses suggesting an inflammatory role of DN T cells in lupus nephritis and SLE patients (215).

Besides the enrichment of Th17 and DN T cells, an increase of T follicular helper cell subpopulation has been associated with SLE. Tfh cells were found in lymphoid aggregates and ectopic germinal centers in nephritis lesions as well as in circulation and correlated with antibody concentration and frequency of plasmablasts (216,217). Tfh cells are essential for B cell maturation, differentiation and antibody production. The interaction of Tfh and B cells typically occurs in GC but can also happen at extrafollicular sites. Tfh cells provide survival and differentiation signals which is crucial for B cell selection and maturation into memory B cells and long-lived plasma cells. In autoimmune diseases, autoantibody production seems to originate from GC reactions as well as from extrafollicular pathways (201,218).

Additionally to Tfh cells, T follicular helper-like cells, also called T peripheral helper cells (T_{PH}) have been identified in blood and tissue of SLE patients and correlate with disease activity and B cell differentiation (**Figure 7**) (219–221). Circulating T_{PH} cells express high levels of IL-21, IL-10, succinate and are enriched in IFN-inducible and cytotoxic-related genes (219–221). T_{PH} express high levels of PD-1 but are CXCR5 negative. T_{PH} share similar functions to Tfh cells by helping B cell differentiation towards antibody producing cells. However, T_{PH} help seems to be limited to memory B cells and possible atypical memory B cells (222).Tfh and T_{PH} cells co-exist in blood and inflamed tissue. T_{PH} are localized extra-follicular while Tfh are localized in the B cell follicles (222).

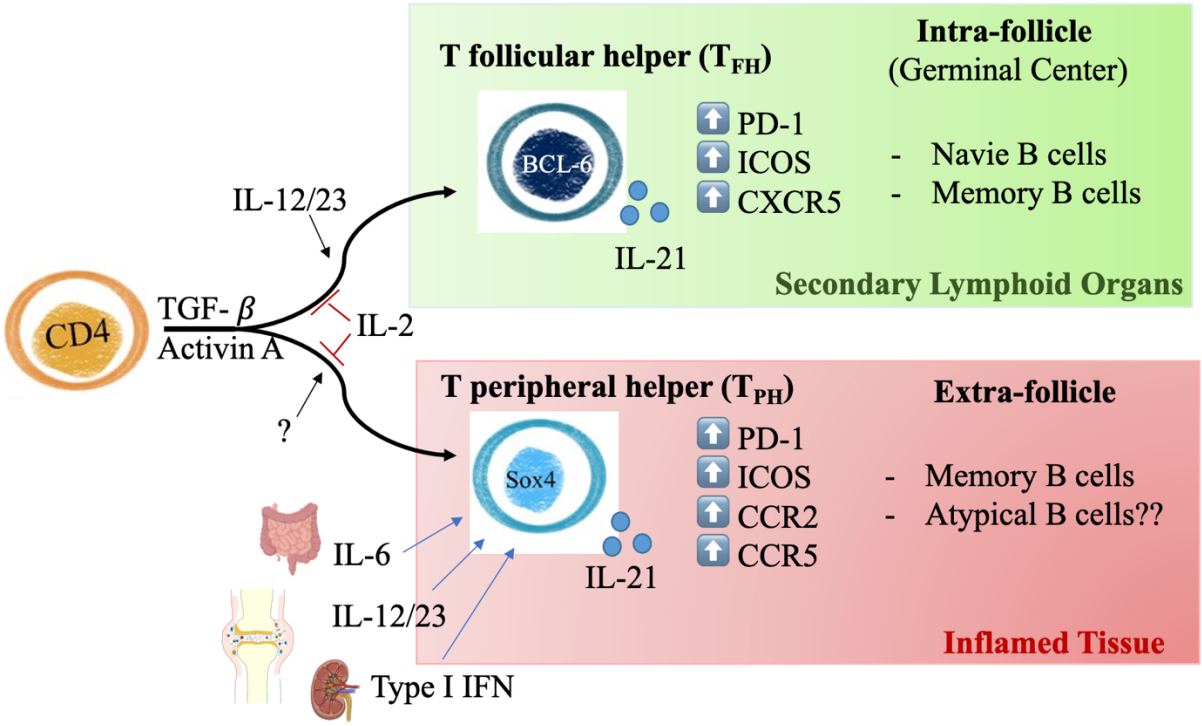


Figure 7: *Differentiation pathways of T follicular (Tfh) and T peripheral (T_{PH}) helper cells (adapted from* (222)*). While Tfh localize intra-follicular and can give help to naïve and memory B cells, T_{PH} localize extra follicular in the inflamed tissues and are limited to memory B cells.*

1.2.8 Lupus arthritis

Lupus arthritis is one of the most common and early manifestations of SLE and has a significant burden on the patient's life quality (223). The clinical presentation of joint involvement differs and ranges from joint pain without erosions or deformations to an erosive joint inflammation leading to severe functional disabilities.

Typically, SLE patients exhibit a transient or persistent often symmetrical joint inflammation in the small proximal interphalangeal and metacarpophalangeal joints of the hand and/or knees, but can occur in any joint of the body (87). Generally, arthritis in SLE patients is transient, migratory and reversible. However, in some cases, it may take a more chronic course, which may resemble rheumatoid arthritis (RA).

Classically, two subtypes of deforming joint involvement are described in SLE. A very rare form is called Rhupus and leads to a RA-like pattern of erosive arthritis in the presence of RF and/or ACPA and anti-RA33 antibodies (see chapter above ACPA in SLE)(129). The other form is called Jaccoud's arthropathy and leads to hand deformities due to repeated tenosynovitis episodes and is associated with longer disease duration and anti-SSA/Ro, RF and antiphospholipid antibodies (223–226). However, only a small proportion of lupus arthritis accounts for these subtypes (227).

Little attention has been given to the pathogenic mechanisms of lupus arthritis, even though 90% of SLE subjects encounter joint inflammation, pain and morning stiffness (223). Histologic studies investigated the properties of synovial fluid and membrane of SLE patients. SF typically includes low white blood cell counts with a predominance of lymphocytes and a normal viscosity (228). In synovial membrane, Goldenberg et al. found perivascular mononuclear cell infiltrates as well as fibrin deposits on the surface(223). In comparison to RA, SLE synovial membrane contains less lining cell hyperplasia and mononuclear cell infiltrates (223,229,230).

A recent study examined the global gene expression profiles in the synovial tissue (ST) of a small SLE cohort. Upregulated genes in ST of SLE patients included type I IFN genes while genes in extracellular matrix homeostasis were downregulated. In comparison to RA, SLE ST showed less infiltrating T and B cells (231). Moreover, Eilersten et al. found a correlation between ongoing arthritis and elevated levels of IL-6 in serum of SLE patients (232). This observation was confirmed by Ball et al. and IL-6 levels correlated with clinal and ultrasound

measures for arthritis disease activity (233). They also measured cytokine levels in SF of 3 SLE patients and found elevated IL-6 levels and lower levels of BAFF and TNF (231). However, due to the low sample volume, future studies have to confirm this finding. IL-6 is synthesized locally and predominantly by monocytes, fibroblasts and endothelial cells and less frequently by lymphocytes. It is a pleiotropic cytokine and is secreted in an acute phase response (234).

Indeed, IL-6 inhibition by Tocilizumab was able to improve arthritis in all 7 lupus arthritis patients (235). However, the presence of IL-6 in SF has still to be confirmed and the underlying pathogenic mechanisms are still poorly understood, even though joint involvement represent a common manifestation in SLE. Thus, there is a need to investigate the pathogenic mechanism underlining synovitis in SLE.

1.3 B CELL TARGETING THERAPY

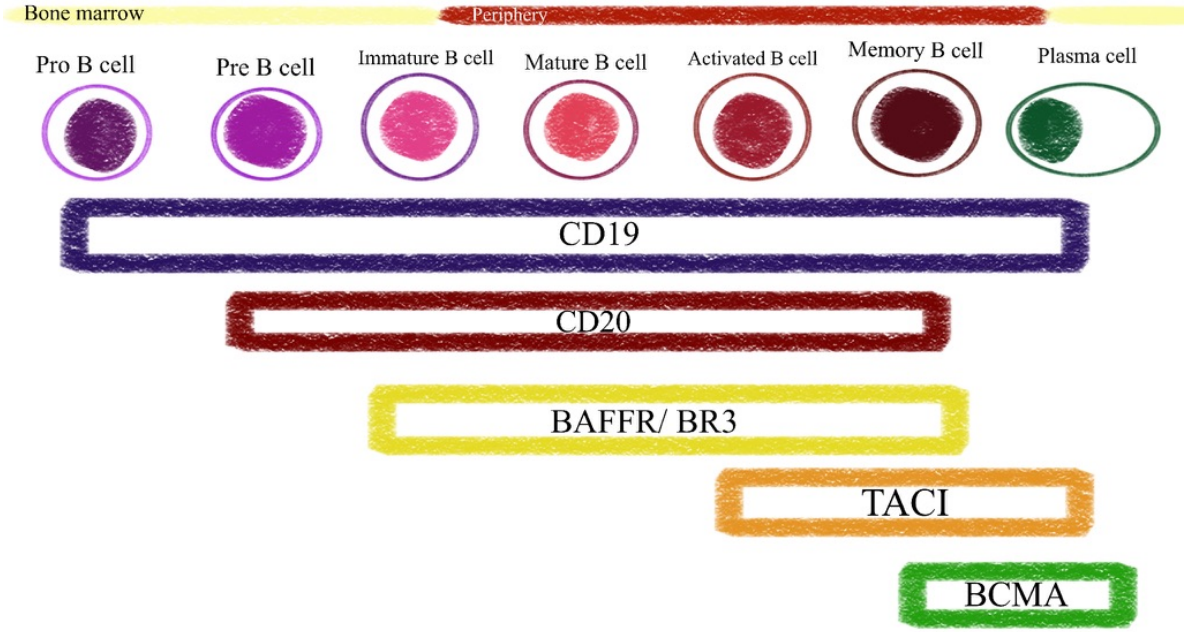


Figure 8: *Expression of CD19, CD20 and the three different B cell activating factor (BAFF) receptors on different B cell development stages. The figure was adapted from* (236) *and* (237).

1.3.1 Rituximab

Rituximab is a chimeric monoclonal antibody specific for the transmembrane protein CD20 and was first developed and approved in 1997 for the treatment of CD20-positive-B-cell-non-Hodgkin lymphoma (184). More recently, rituximab found use in potentially B cell-mediated autoimmune diseases. In 2006, the Food and Drug Administration (FDA) approved the drug for RA patients, who failed TNF antagonist therapy (238). Nowadays rituximab is used for treating several autoimmune diseases, such as MS and SLE, in patients which failed standard therapies (239,240). Besides rituximab, other therapeutic antibodies (biosimilars) directed against the CD20 molecule and towards other B cell targets have been developed and are currently available on the market (241).

The mechanism of action of rituximab include (238)**:**

- Induction of apoptosis
- Complement dependent cytotoxicity
- Antibody dependent cellular cytotoxicity

1.3.1.1 Rituximab treatment in SLE

The first use of rituximab in SLE was reported in 2002 (242). Since then, several case reports described a beneficial effect of rituximab in SLE patients (243–245). Since B cell dysfunction is a hallmark of SLE, rituximab seemed to be a promising drug for the disease. Consequently, two randomized control trials were performed. The EXPLORER and LUNAR study explored the use of rituximab in moderately to severely active extrarenal as well as lupus nephritis patients (246,247). Surprisingly, the clinical trials did not meet their primary and secondary endpoints and no differences could be detected between rituximab and placebo treated SLE patients (246,247). These discouraging results could have originated from the pitfalls of the trial design, limitations in assessing the various clinical end points and the heterogeneity in manifestation of the disease (248,249). Despite the failure of those clinical studies, rituximab is still used by rheumatologists as off-label treatment for patients who are refractory or intolerant to conventional therapies (248).

1.3.1.2 Function and expression of the CD20 molecule

Rituximab binds to the surface molecule CD20. CD20 is expressed by the majority of B cells in different B cell states starting from pro-B cells to memory B cells (**Figure 8**)(236), but it is lacking on pre-B cells, terminally differentiated plasmablasts and plasma cells. CD20 can interact with various proteins including MHC molecules, CD40 and BCR (236). The function of CD20 still remains unclear. Several mice studies tried to shed light on the function of CD20, however, CD20 knock out mice presented a relatively mild phenotype. Despite that, the data suggested that CD20 is required for optimal T-independent and T-dependent humoral immunity as well as for efficient BCR signaling (238). In humans, Kuijpers et al. described a patient lacking the CD20 protein due to a unique mutation. The absence of CD20 resulted in a decreased number of memory B cells in peripheral blood, reduced IgG antibody levels and isotype switching. Additionally, this patient showed impaired antibody formation after vaccination, suggesting that CD20 on B cells is involved in B cell maturation (238). CD20 is mainly expressed by B cells, although a subpopulation of T cells expressing CD20 has been previously described (250,251). Such CD20+ T cells are enriched in MS patients in comparison to healthy donors. They exhibit a greater migration capacity and the amount of CD20+ T cells in CSF correlates with disease activity, indicating a potential role in MS (252,253).

1.3.1.3 Effect of Rituximab on lymphocytes

The effect of rituximab on B cells has been studied in the context of various diseases and might differ depending on the pathogenic mechanism of the disease.

In response to rituximab, the number and frequency of CD19+ B cells decrease (254–256). B cell repopulation mostly occurs around 6-9 months after treatment and normal B cell counts are reached by around 12 months with full mature B cell recovery. Regeneration of memory B cells is delayed and does not reach baseline levels after two years (255,257). Naïve, memory and immature B cells are rapidly depleted. However, certain memory B cells seem to be more resilient to rituximab treatment or tissue-resident memory B cells enter the circulation, since more than 80% of residual B cells show a memory or plasma cell precursor phenotype (245).

In lymph node biopsies of RA patients, follicular B cells, unswitched memory and naïve cells are depleted while CD27+IgD− switched memory B cells persist in lymph node biopsies after rituximab treatment (257). Similar results were observed in BM samples after rituximab treatment. Short-term changes included depletion of naïve and unswitched memory B cells, while the decrease of memory population was long-term. Pre-switched memory B cells were not as affected as pre-germinal center B cells by B cell depletion (258). Autoantibody levels drop in some patients upon rituximab treatment, in others they are maintained and clinical improvement is not necessarily linked to the absence of serum autoantibodies (259). Besides B cells, rituximab influences frequencies of T cells, NK, cells and macrophages phenotypes (241).

1.3.1.4 Rituximab treatment and clinical outcomes

Although some memory B cells, plasma cells and autoantibody levels persist during rituximab treatment, abnormalities in B cell homeostasis are improved one year post-rituximab in SLE patients, with a decreased proportion of autoreactive memory B cells after treatment (260). Differences in relapse vs remission were reported by several different groups. An earlier and rapid repopulation can be found in patients with early relapse. In general, relapsing was associated with high anti-dsDNA antibody levels and increase of IgD−CD27^{hi} plasmablasts (261). Insufficient deletion of autoreactive memory B cells might result in earlier relapsing. Indeed, a higher portion of memory B cells and lower frequency of immature B cells during repopulation correlated with earlier disease relapses in SLE patients (241).

In RA patients, clinical response to rituximab was related with the depletion of memory B cells (CD19+CD27+) in PB and BM (262,263). This suggests that the outcome of B cell depleting therapy might depend on the balance between protective and pathogenic B cell populations (264).

Importantly, the effect of the autoimmune associated B cells subset - 'atypical' memory B cells/ABCs - upon rituximab therapy has not been studied yet. ABCs express CD20 on their surface but could also be more resistant than their relatives – memory B cells. Interestingly, after rituximab therapy, BAFF increases in in SLE, RA and primary Sjögren's syndrome (265–267). Patients with expanded autoantibody profile and raised BAFF levels at baseline had shorter clinical responses and BAFF levels were higher in relapsing SLE compared to patients at disease remission (261). This suggests, that BAFF inhibition together with rituximab might result in higher rates of clinical response.

1.3.2 Belimumab

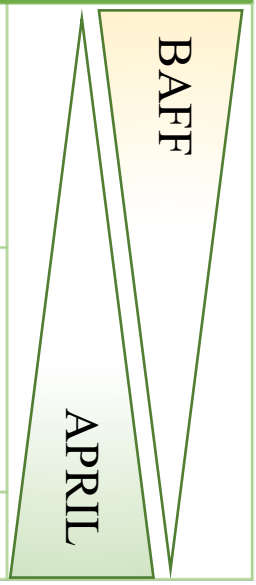
Belimumab is a fully human IgG1 lambda monoclonal antibody directed against the B cell activating factor (BAFF). It is the first approved and only targeted biological drug currently available for SLE (268).

1.3.2.1 B cell activating factor and its receptors

BAFF (BLyS, TALL-1,CD257, TNFSF13B) is a key survival, differentiation and activation factor for B cells and belongs to the TNF family(237). It is produced by monocytes, macrophages, dendritic cells, T cells, osteoclasts and stromal cells (237). It can exist in both a soluble and a membrane bound form (269). Three different receptors bind BAFF and are present on several immune cell types. In B cells, the three receptors are expressed during different stages of B cell development fulfilling a diverse set of functions (**Table 2 and Figure 8 (237)**).

While BAFFR is expressed from transitional B cell stages to mature B cells stages, it is downregulated in plasma cells (270). TACI and BCMA additionally bind the protein A proliferation-inducing ligand (APRIL). Interestingly, the survival of switched memory B cells have been shown to be independent from BAFFR or TACI (**Table 2**). In contrast, follicular, marginal zone and transitional B cells depend on BAFF-induced survival signals and BAFF is essential for early B cell differentiation (237).

***Table 2**: BAFF Receptors expression, function and APRIL and BAFF binding hierarchy*

Receptor	Expression on B cell subsets	Function	APRIL-binding
BR3/BAFFR (BAFF receptor)	IgM+ immature B cells, transitional B cells, naïve B cells, memory B cells, marginal zone B cells, follicular B cells, age/autoimmune-associated B cells, activated/memory B cells Exclusion: plasma cells and centroblasts	- B cell activation, survival and differentiation	
TACI (T cell activator and calcium modulating ligand interactor)	Transitional 2 B cells, activated B cells, Plasma cells, marginal zone B cells, switched-memory B cells, age/autoimmune-associated B cells	- triggering B cell class-switching - decoy receptor function to neutralize BAFF - survival of plasma cells	
BCMA (B cell maturation antigen)	Plasma cells, activated B cells	Survival of long-lived plasma cells	

1.3.2.2 Pathogenic role of BAFF in SLE

SLE patients overexpress BAFF and BAFF levels correlate with disease activity and autoantibody levels (269,271).The pathogenic potential of elevated BAFF has been further explored in mice. Administration of BAFF resulted in B cell expansion and increased antibody levels. Moreover, BAFF-transgenic mice established a severe B cell hyperplasia and lupus-like illness which was categorized by the presence of anti-nuclear autoantibodies and immune complex deposits in the kidneys (272,273). Dysregulation of BAFF expression has been shown to provoke autoimmunity in animal models. Increased BAFF levels can be detected in active and relapsing SLE patients after rituximab treatment, suggest a potential pathogenic role of BAFF in SLE (261). Overall, BAFF inhibition represented a potentially important therapeutic target in SLE.

1.3.2.3 Belimumab in SLE

Belimumab neutralizes BAFF by binding soluble BAFF and therefore blocking it from binding to its receptors. Two multicenter phase II trials (BLISS-52 and BLISS-76) met their primary end points, however, with rather modest success (274,275). Most importantly, belimumab treatment achieved long term success with confirmed efficacy and steroid-sparing effect (276). A recent longitudinal study followed patients on belimumab treatment up to 13 years. The patients who initially demonstrated a satisfactory response continued to tolerate belimumab and long-term disease control was provided (276). However, belimumab might not be beneficial for all SLE patients. 40% of SLE patients in the clinical trials did not

reach a meaningful clinical effect and given the costs of the treatment, belimumab is rarely given as first-line treatment(276).

1.3.2.4 Effect of Belimumab on B and T cells

The effect of belimumab on B cell subsets has been demonstrated in early longitudinal studies (277,278). The total number of B cells decreases after 3-5 months. Interestingly, the effect on B cell subsets seems to separate into two groups. While the frequency of transitional and naïve B cells reduces early on, the frequency of unswitched-memory and plasmablasts is affected later on by the treatment. Memory B cells seem to be more resilient towards BAFF inhibition (277,278). A detailed investigation of the changes in B cell phenotypes upon belimumab treatment in combination with clinical response is missing in this field.

The absolute numbers of T cells seems to not be affected by BAFF inhibition, even though BAFFR and TACI are expressed on T cells subsets (277,278). However, a detailed analysis of T cell phenotypes in SLE patients treated with belimumab is still missing.

2 RESEARCH AIMS

The overall aim of the thesis is to study lymphocytes and autoantibodies involved in the pathogenesis of RA and Lupus Arthritis as well as alterations of lymphocyte phenotypes after B cell depletion in SLE.

Specific aim of the studies included in the thesis:

In **Paper I**, we studied alterations of age associated B cell and specific T cell subsets longitudinal after rituximab treatment in SLE patients.

In **Paper II**, we examined changes of B and T cell phenotypes after belimumab treatment using mass spectrometry and correlated them with clinical responses.

In **Paper III** we explored the cytokine profile associated with T cell responses and cellular compartments in synovial fluid of lupus arthritis patients.

In **Paper IV** we investigated the citrulline reactive B cell repertoire in peripheral blood of RA patients in order to assess the BCR diversity as well as studying their antigen-recognition and effect *in-vivo*.

3 MATERIALS AND METHODS

This section gives a general overview and highlights the strengths and limitations of the main methods in the thesis. A more detailed description of the exact methodology can be found in the corresponding papers.

3.1 ASSESSMENT OF DISEASE ACTIVITY IN SLE PATIENTS

In order to measure disease activity, responsiveness and quality of life of SLE patients, clinical trials and long-term observational studies make use of assessment tools. For this purpose, several indices have been established and revised.

The physician global assessment (PGA) is a visual analog scale that assesses disease activity over 2 weeks and is a sensitive measure to score the patient's overall condition (279).

SLE Disease Activity Index (SLEDAI) is one of the most common global indices to assess disease activity of SLE patients. SLEDAI was first developed in 1986 and described in 1992 and evaluates 24 items during the past 10 days. Currently, three different revised versions of the original SLEDAI are available: SLEDAI-2K, SELENA-SLEDAI, MEX-SLEDAI (90). Unfortunately, the index is not graded, you either have or don't have the assessed manifestation, thus it cannot identify small improvements or worsening of the disease.

The British Iles assessment group (BILAG) is another more elaborate and graded index which is commonly used and evaluates organ specific severity over the past one month (90).

Due to the benefits and disadvantages of each index, recent clinical trials use a combination of assessments as primary outcome in order to investigate efficacy of new biological targets. The SLE responder index (SRI) combines SLEDAI for global disease activity, BILAG for organ specific changes and Physician Global Assessment for validity (279).

Lupus low disease activity score (LLDAS) is a tool that determines the state of low diseases activity. LLDAS should be the aimed for as treatment outcome. It is defined by low SLEDAI-2K, PGA, prednisone dose as well as no activity in major organ systems, no new SLE activity and a well-tolerated dose of immunosuppressive drugs (280).

In **Paper II,** we used SRI to evaluate the response to therapy and LLDAS to define low disease activity.

Unfortunately, indices are not commonly used in routine care. It is difficult to ascertain disease activities from retrospective samples and to quantitatively monitor clinical care.

3.2 SAMPLE PREPERATION

Freezing, storage conditions and thawing procedures can influence cell viability, sample composition and gene expression (281–283). Several studies have optimized these procedures and highlighted that several factors are important for good cell viability and sample quality (282–287,287–291). These include the right cell density, the right cryopreservation medium, and an optimal cooling rate(292). Storage time in the liquid nitrogen tank and fluctuation in storage temperature can also influence the sample (289). The thawing procedure itself, also has a substantial impact in recovering viable cells from frozen samples (283,287,288). Isolation and thawing of peripheral blood mononuclear cells (PBMC) from SLE or synovial fluid mononuclear cells (SFMC) is challenging due to the increased clumping of cells, which can be improved by the addition of the enzyme benzonase (288). Clumping of cells results from an increase of activated or apoptotic cells. Isolation and cell recovery from frozen SLE sample are especially tricky, since many of them are lymphopenic (293).

Moreover, thawing and freezing cycles affect the concentration of cytokines and can alter the composition in serum and acellular synovial fluid, thus it is of great importance to use previously unthawed samples for cytokine analysis and to thaw them slowly on ice (282,284).

3.3 MULTICOLOR FLOW CYTOMETRY

Flow cytometry is a frequently used, powerful application which rapidly analyses size, complexity and protein expression of millions of single cells or particles in suspension. Single cells pass through one or multiple laser beams and emit light to detectors. With the help of fluorochrome-conjugated antibodies specific for extra- or intracellular molecules, different populations and features can be evaluated. Currently, advanced instruments can measure up to 30+ markers (294).

In this thesis, flow cytometry is used to immunophenotype peripheral blood and synovial fluid mononuclear cells as well as measuring of cytokines. In **Paper IV**, we applied a novel method to identify antigen-specific B cells in peripheral blood of SLE patients with the help of fluorescently labelled tetramers. Thus, the following parts describe the general principle of flow cytometry, different applications and discusses the pros and cons of the application.

3.3.1 General Principle of Flow Cytometry

A flow cytometer consists of three different parts: fluidics, optics and electronics. First, the sample is injected into a stream of sheath fluid inside the instrument. Due to pressure differences, the sample stream is aligned in the core of the flow stream which forces the

particles to arrange in a single fashion. This ensures the passing of only one particle at a time through the laser beam. The higher the sample flow rate, the wider the sample stream and the higher the possibility to have more than one cell passing through the laser beam at the same time. Once the cell passes through the laser beam, light is scattered and captured by detectors. The forward and side scatter are used to measure size and granuality/complexitiy of a cell. If the cell is labelled with antibodies, the fluorochrome of the antibody is excited by a certain laser beam and emits photons to a detector. The photons are then converted into electrons and send a signal to the computer where the cells are shown as dots on a dot plot or in a histogram and can be analyzed (295).

3.3.2 Advantages of Flow Cytometry

It is a fast way to analyze different types of cells or particles in a fluid and can even be done in high throughput. The applications in which flow cytometry can be used seem endless. Microbiologists use it to identify bacteria, hematologists to analyze platelets and microparticles, oncologists to look at chromosome abnormalities, and immunologists to deep phenotype immune cells. Using cell sorters, specific subpopulation of cells can be isolated and further analyzed for functional assays or sequence analysis in bulk or at single cell level.

3.3.3 Disadvantages of Flow Cytometry

One of the biggest disadvantages of flow cytometry is spectral overlap of emitted light from the fluorochromes. Different fluorophores are excited by different lasers and emit light of different wavelengths which travel through selected band pass filters and reach the corresponding detector within a flow cytometer. However, emission spectra of different fluorochromes can overlap and spill over to other detectors, which can affect the strength of the signal and separation of two different markers can be difficult. Thus, single stains, which measure the fluorescent signal in all channels are used to calculate spill over value or so-called compensation. The values are then subtracted from the sample in order to get better separation of the populations (296) and consolidated in a matrix which can be manually modified. Besides compensation itself, it is important to carefully design the panel used for the experiment, to minimize spectral overlap of fluorochromes and to maximize detection and separation of populations. Low or unknown antigens should be stained with bright fluorochromes while high antigen expression should use dim markers. It is also important to use a live/dead stain since dead cells increase the background and can falsify results. Antibody titration reduces non-specific binding and increases sensitivity (294).

In general compensation is complex, and incorrect compensation can lead to false conclusions and results. It is essential, to carefully design the experiment, prepare the right controls and to check the compensation during analysis.

Over the last couple of years, a new technology called spectral flow cytometry was designed to measure the full emission spectrum of a fluorochrome and to create so called “spectral fingerprints”. During the analysis, the spectra of the sample is unmixed in order to analyze single markers. This enables the distinguishing of fluorochromes which are normally very close to each other, permitting the use of several spectrally similar markers simultaneously, which would have been impossible with traditional flow cytometry (294).

3.3.4 Cytokine Bead Arrays

Cytokine Bead Arrays (CBA) are part of the new multiplex technologies, which are able to detect several different cytokines or other substances in one sample at the same time. The beads contain integrally different dyes and sizes and are covered with antibodies specific for its desired cytokine (297). This allows simultaneous detection of up to 30 multiple analytes in one single sample. For **Paper III**, we decided to use the preconfigured BD Th1/Th2/Th17 CBA Kit, which included the cytokines IFN-γ, TNF, IL-2, IL-4, IL-10, IL-6 and IL-17A (298).

In comparison with ELISA and Western Blots, CBA has a low sample volume requirement (25-30ul) and is less time consuming. CBA can also be analyzed using any kind of flow cytometer while other multiplex assays, such as Luminex, require special detection systems (299). The protocol is easy to follow and the flow cytometry analysis rather fast. Also, the corresponding software for calculating the concentrations is easy to handle. However, CBA display a lower sensitivity and a higher level of detection than ELISAs. Moreover, CBA are standardized for plasma and cell supernatant, but not for synovial fluid and Cell debris may interfere with the results and might have been the reason why we did not detect IL-2, IL-4, IFN-γ and TNF in our samples. In a study by Richens et al. Luminex kits were found to be more reliable and reproducible as CBA kits (294).

3.3.5 Mass cytometry

In **Paper II**, we used mass cytometry to study longitudinal changes of B and T cell phenotypes from peripheral blood of SLE patients after treatment with Belimumab. Mass cytometry combines the principles of flow cytometry and time-of-flight mass spectrometry. Instead of fluorochromes, antibodies are coupled to metal isotopes and detected using a mass spectrometer. Due to the use of metal isotopes, compensating is redundant since there is no

spectral overlap or autofluorescence. Compared to flow cytometry however, mass cytometry is expensive, and the acquisition rate is much lower (300,301). Here, they were run at a core facility.

3.3.6 Analysis of Flow Cytometry Data

Multiple commercially available computer software are available for the analysis of flow cytometry data (295). Due to the increase of parameters, the analysis became more complex, challenging and time consuming. Usually, flow cytometry data is displayed two-dimensional, by plotting two colors against each other. New tools used originally for sequence analysis such as t-Distributed Stochastic Neighbour Embedding (tSNE) and principal component analysis (PC) are of great asset nowadays to visualize and evaluate the enormous data sets generated by flow and mass cytometry (295,302).

3.4 CAPTURING ANTIGEN-SPECIFIC B CELLS

In 1987, Hayakawa et al. were the first group to analyze antigen-specific B cells towards the highly fluorescent protein PE using flow cytometry in PE-immunized mice (303). Since then, several procedures were developed and various molecules such as recombinant and synthesized proteins, lipids, haptens, polysaccharides and virions have been utilized to detect antigen-specific B cells by multi-color flow cytometry(304).

In **Paper IV**, we constructed citrulline- specific B cell tetramers in order to capture and analyze citrulline specific B cells. Newman et al.(305) were the first to use peptide-coupled B cell tetramers to identify PE- specific B cell in immunized mice. The idea of using tetramers originated from the previously developed methodology of T cell tetramers where HLA molecules are coupled to a fluorescently labelled streptavidin (SA) core in order to enable direct quantification of antigen-specific T cells. B cell tetramers improve avidity of the BCR, brightness of staining and have been found to be more sensitive than monomers (306). In our experiments, we separately coupled synthesized, biotinylated, circular citrullinated peptides from filaggrin (Cfc1/CCP1) or alpha enolase (CEP-1) to a PE labelled streptavidin (SA) core.

However, in contrast to analyzing antigen-specific T cells, a significant B cell population will bind not only to the epitope of interest, but also to epitopes originating from the tetramer complex (SA, fluorochrome, biotin) (307). Two different approaches have been established, to reduce this problem. Using the same tetramer with two differently labelled fluorochromes and selecting for double positively stained cells, fluorochrome-reactive B cells can be excluded (304,308). However, the population will still be contaminated by B cells binding SA. Thus, the second tactic incorporates a non-specific control tetramer, the so-called decoys,

in the procedure. Decoys are labelled with an additional fluorochrome to alter the emission spectrum and are excluded during cytometric analysis to distinguish irrelevant B cells from the B cells of interest (307). For the decoy tetramers in **Paper IV**, the arginine version of our peptides was coupled to our decoy tetramer and the SA-PE core was conjugated to AF647 (**Figure 9**).

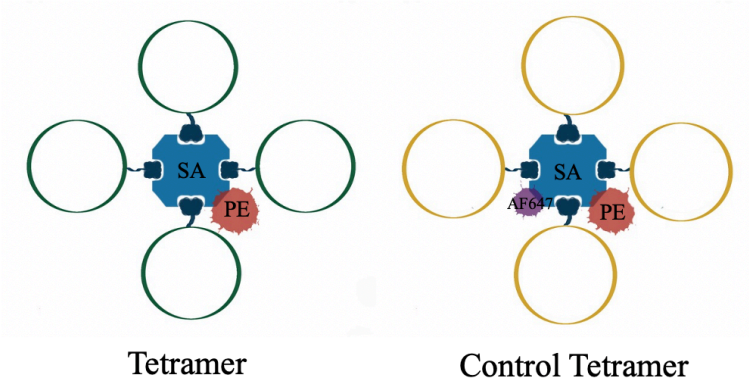


Figure 9: Representation of the B cell tetramers constructs used in Paper IV*. The B cells tetramers core consists of a streptavidin (SA) and coupled fluorochromes; PE for the antigen-tetramer and PE and AF647 for the control tetramer (decoy). Biotinylated circular citrullinated (in green) and circular arginine (in yellow) peptides from either filaggrin or alpha enolase.*

One of the biggest challenges in detecting antigen specific cells, is their rare frequency in circulation. Antigen- specific B cells are typically estimated to occur at a frequency of 0.05 to 0.005% in the normal repertoire (309). Thus, magnetic enrichment techniques are of great importance during the procedure (304). Concentrating the cells can either be done for a specific fluorochrome or specific cell subset prior flow cytometry analysis. We enriched our cells for the fluorochrome PE using magnetic beads. This enrichment step allows significant more cells to be analyzed in a shorter time period.

Other things which have to be considered are bright fluorochromes (PE) used for tetramer labelling as well as the markers for phenotyping which should have as little spill over into the tetramer channel as possible (304).

4 RESULTS AND DISCUSSION

In **Paper I and II,** we investigated the longitudinal alteration of T and B cell subsets in SLE patients undergoing B-cell targeting therapy. This is a useful tool to understand the mechanism of the treatment as well as identifying responders vs non-responders.

4.1 INFLUENCE OF RITUXIMAB ON DOUBLE NEGATIVE (DN) AND AGE/AUTOIMMUNE- ASSOCIATED B CELL (ABC) SUBSETS

In **Paper I**, we studied primarily the effect of rituximab, and chimeric anti-CD20 antibody, on double negative B cell subsets as well as on T follicular/follicular-like T cells subsets in 10 lupus nephritis and 5 non-lupus nephritis patients using multicolor flow cytometry.

SLE is characterized by enrichment of certain B cell phenotypes such as double negative B cells, plasmablasts and ABCs (CD21-CD11c+) which correlate with disease activity (182,185). In our SLE study cohort, DN accounted for 12% of CD19+ B cells at baseline, of which 20% expressed CD11c+ without CD21 (ABCs). A small but distinct population of plasmablasts (CD27++CD38++) was observed in our patients reaching from 0.14-5% of CD19+ cells.

First, we wanted to assess the efficacy of rituximab in depleting B lymphocytes in our patient cohort. B cell frequency reduced from a median of 11.3% (IQR: 5.55-21) to 0.74% (IQR: 0.67-0.94) after 2-4 months. Three patients retained a B cell frequency of 2-5%. Variability in B cell depletion has been previously described in SLE patients and might result from polymorphism in the Fc gamma IIIa receptor or possible complement defects (260,310,311). Similar to previously published results (245,255,312), the frequency of B cells predominantly decreased in the naïve (IgD+CD27-) B cell subset while the frequency of the remaining B cells increased, i.e. the plasmablasts (CD38++CD27++,9%), memory (IgD-CD27+, 32%) and DN (IgD-CD27-, 14%).

Repopulation of B cells has been shown to occur around 6-9 months after rituximab treatment (255,313). In our study, an increase of naïve B cells was observed from around 6 months onwards. After approximately one year, the naïve B cell population accounted for ~80% of CD19+ B cells. At baseline naïve B cells accounted for a median of 70% (IQR: 61-77) of the total B cell population. A distinct regeneration pattern of B cell subtypes after rituximab treatment has been previously described (255,314). Immature B cells repopulated first, followed by naïve B cells. In contrast, memory B cells were delayed in repopulation and were reduced even after two years (255,314). In our cohort, memory B cell frequency were reduced

after one year, however without significance. Since we only had a small sample size and less patients in the later follow up (5 patients in the >longer timepoint), it is difficult to calculate statistical power. Upon rituximab treatment, the proportion of DN in CD19+ B cells first increased after 2-4 months but then significantly decreased by 6.3% (p=0.01) after 9-10 months compared to 2-4 months. The effect of rituximab on DN frequencies and numbers has been previously reported (312,314).

The DN enrichment in SLE patients originates from the increase of so called DN2 cells, a subset which was described by Jenks et al. (185). They distinguished two DN subsets using the surface markers CXCR5 and CD11c into: DN1 (CXCR5+CD11c-) and DN2 cells (CXCR5-CD11c+) B cells. Besides CD11c, DN2 cells are negative for CD21- and CXCR5- and express the transcription factor T-bet. Thus, they are likely to resemble ABCs. We decided to include the marker CXCR5 in our panel together with the typical ABC- markers CD11c and CD21. Since we included the marker later in the study, CXCR5 was only measured in 10 of the 15 patients, which could have influenced the statistical tests **(Figure 10).** Baseline DN2 frequencies correlated with ABC frequencies (r=0.8, p=0.001). After 2-4 months of rituximab treatment, we observed a significant decrease of DN2/ABCs (ABCs: 13%, p= 0.03; DN2: 14%, p=0.01). ABCs repopulated after approximately 9 months. DN2 increased in some patients, while in others the frequency stayed low. Interestingly, the total frequency of CD11c+CD21- did not change significantly after treatment indicating the need for multiparameter approaches when examining ABCs.

In contrast to DN2 and ABCs, DN3 and DN CD21-CD11c- B cells increased significantly (CD21-CD11c-: 24%, p=0.002, DN3: 6.7%, p=0.03) after 2-4 months of rituximab treatment **(Figure 10).** DN3 cells are suggested to resemble early memory precursor and a recent study using single-cell transcriptome analyses indicated a relationship between DN3 cell and classical memory B cells (315). Thus, DN3 cells might be more resilient towards rituximab treatment, similar to classical memory B cells.
Surprisingly, the frequency DN1 and CD11c-CD21+ B cells did not change significantly. As DN3 cells, DN1 cells have been suggested to be early memory precursors (315).
After approximately one year, we could also observe a significant expansion of DN CD11c+CD21+ cells (6%, p=0.007). DN4 B cells (CXCR5+CD11c+) increased non-significantly after 2-4 months by 11% (p=0.07). Not much is known about DN4 cells. Stewart et al. revealed a high frequency of IgE in the DN4 cells (315), however further research is needed to confirm the findings.

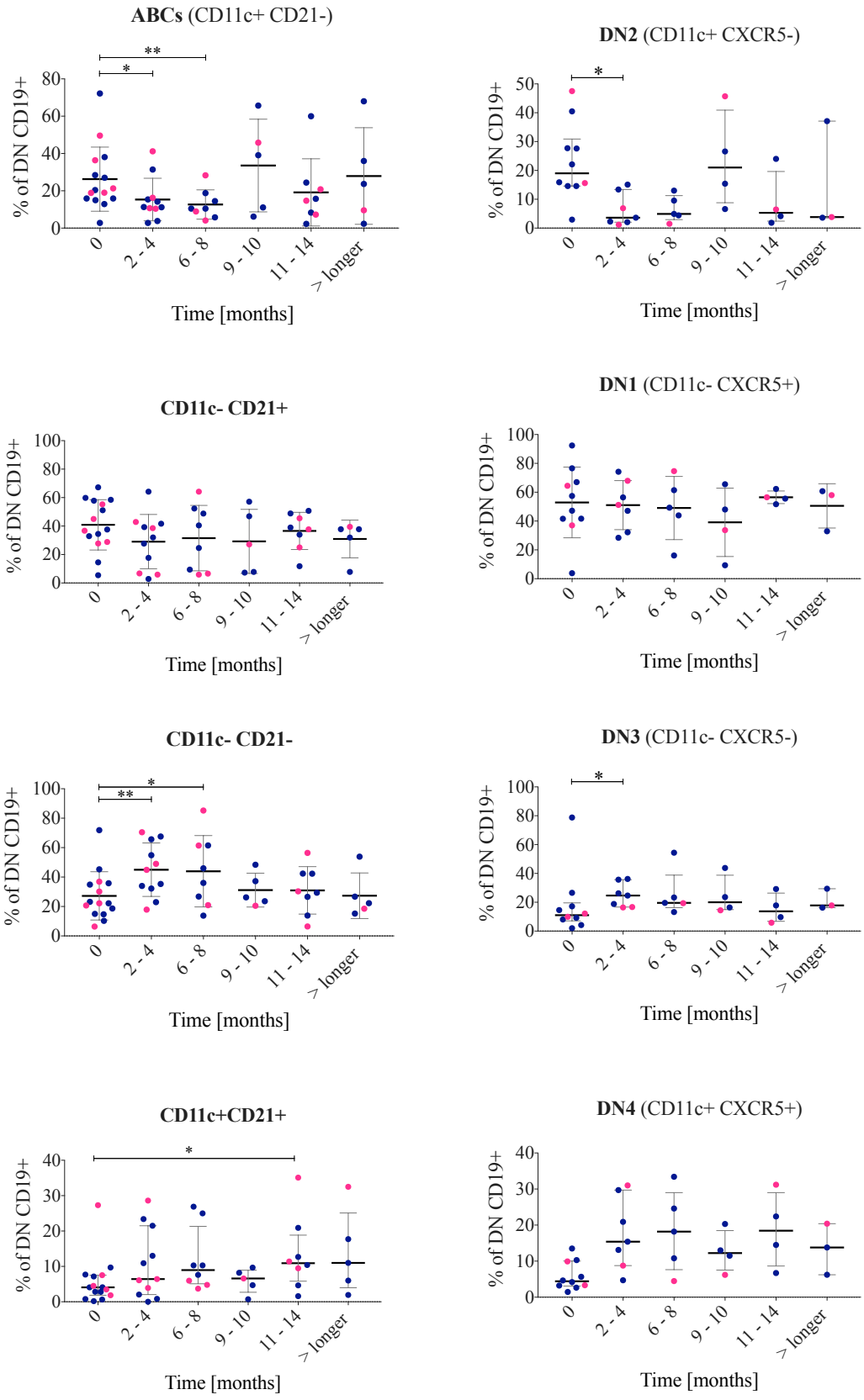


Figure 10. *Impact of rituximab treatment on the frequency of double negative B cell subsets. DN B cells were gated using the markers CD11c and CD21 while in DN1, DN2, DN3 and DN4 subsets were divided using CXCR5 and CD11c. Lupus nephritis patients are indicated in blue and non-lupus nephritis patients in pink.*

Of note, the biggest limitation of our study is, that we did not measure the absolute count of B cells. Hence, we were only able to analyze frequencies of the remaining B cells and are unable to draw firm conclusions of any reduction of B cell subset counts.

To conclude, the frequencies of ABCs and DN2 cells diminished already after 2-4 months after rituximab therapy in SLE patients and repopulate in average after 9 months. Depleting of ABCs/DN2 might be beneficial for the patients, since DN2/ABCs can quickly differentiate into antibody secreting cells and are enriched in autoreactive clones (185,200). DN3 cells, which have been describe recently in severe COVID19 patients, seem to be more resilient towards rituximab treatment, however their function remains still unclear (316). Further analysis of the different DN cell subsets have to be performed in order to understand their origins and functions.

4.2 EFFECTS OF RITUXIMAB ON T CELLS IN SLE

B cells have the capacity to produce cytokines, present antigen and interact with T cells through co-receptors. On that account it is likely, that T cells are directly, by expressing CD20 and indirectly influenced by B cell depleting therapy. A small subset of CD20-expressing T cells might also directly be affected by this therapy. A decrease of T cell activation marker such as HLA-DR, CD40L and CD69 as well as an increase in Tregs upon rituximab treatment has been reported and validated in several studies (314,317,318).Here, we investigated the influence of rituximab on T follicular helper cells, T peripheral helper cells, naïve, effector and central memory T cells as well as on T_{EMRA}.

T:B cell interaction is essential for differentiation and survival of Tfh which are enriched in affected tissue and in PB of SLE patients (216,319). A few studies have investigated the response of Tfh to rituximab, with different outcomes. Wallin et al. reported, that frequencies and numbers of Tfh cells and Tfh subsets were not affected in PB nor in lymph nodes on transplant patients receiving rituximab treatment(320).

In contrast, circulating Tfh in neuromelitis optica spectrum disorder were reduced post-treatment (321). For that reason, we were interested to study if Tfh frequency is influenced by rituximab treatment in SLE patients. Surprisingly, the frequency of circulating Tfh cells in peripheral blood of SLE patients was not influenced by rituximab. An imbalance of circulating Tfh, towards the Tfh2 subpopulation has been described in SLE, and to correlate with elevated DN B cells (216). Here, we did not include markers to study subtypes of Tfh

and it might still be possible, that Tfh subtypes might be influenced differently by cytokines and T:B interactions.

A T-follicular-like cell subset has been recently discovered in SF of RA patients (322). Such PD-1^{high} CXCR5- CD4 T cells are enriched also in peripheral blood of active SLE patients and have been associated with relapses and disease activity (217,323). In our study, baseline PD-1^{high} CD4+ T cells did not correlate with SLEDAI but the frequency of PD-1^{high} CD4+ T cells was reduced after treatment. This observation could either result from an indirect effect through dampening of the immune response or a direct effect in which B cells would support the differentiation of PD-1^{high} CD4+ T cells.
Interestingly, two patients with high SLEDAI after 6 months follow up had an increase in the frequency of CD4+ PD-1^{high} cells. Previous finding reported that the frequency of T_{PH} cells diminished in remission and increased during flare (324), highlighting the pathogenic role of PD-1^{high} CD4+ cells.

When analyzing T cell frequencies, T_{EMRA} (CD45RA+ CCR7-) and effector memory (CD45RA- CCR7-) in CD4+ and CD8+ T cells increased slightly after rituximab treatment but returned to baseline after approximately one year. A recent study in anti-neutrophil cytoplasmic autoantibody (ANCA)–associated vasculitis patients demonstrated a reduction of naive (CD45RA+CCR7+) CD4+ T cells and an increase in effector memory (CD45RA–CCR7–) T cells in patients with disease remission(325). Interestingly, Neel at al. also reported a reduction of CD8+ T_{EMRA} after rituximab treatment. We did not observe the same phenomena in our cohort of SLE patients.

Around 3-5% of CD3+ T cells express the CD20 molecule, and are effectively depleted by rituximab treatment (252,253). Since we did not include CD20 in our panel and we did not analyze the absolute number of cells the deletion of these cells might have impacted our results. Further limitations of our study are the low sample size and the reduction of patients during follow up. Rituximab is often given after and together with other standard of care therapies which might have contributed to the patient's aberrant T and B cell composition.

Overall, we could detect a reduction of PD-1^{high} CD4+ T cells after treatment with rituximab while circulating Tfh frequencies were not impacted by rituximab. We still need to perform an in-depth correlation analysis of the clinical data with our immune phenotyping.

4.3 EARLY AND GRADUAL B CELL ALTERATIONS UPON BELIMUMAB TREATMENT

Belimumab is the first biological agent to be approved by the FDA for SLE patients (268). In **Paper II**, we studied the effect of belimumab on T and B cell phenotypes over time and in response to clinical measurements. For this purpose, we obtained blood samples from 23 SLE patients before and during belimumab treatment (from 3 to 6 occasions) and analyzed cell phenotypes using mass cytometry. The clinical response was measured using SRI, SLEDAI-2K, BILAG, PGA and low disease activity by LLDAS (which are described in detail in the method section).

We evaluted our obtained dataset from two different angles:

1. in a biased approach using well-defined cell populations
2. in an unbiased manner by using specific clustering and dimensionality reduction tools.

First, we wanted to explore the B cell diversity in our SLE patient cohort before initiating belimumab treatment. For this analysis, baseline CD20+ B cells were assembled into clusters based on markers and their surface expression levels using tSNE. We could observe a large fraction of memory (CD27+) B cells and pronounced cell differentiation. Clusters outside the typical memory and naïve repertoire gained our attention. Two clusters displayed expression of CD11c+ and low CD21 and CD27, implicating ABCs. In addition, we could also identify two small but unique subpopulations expressing CD57 or CD14+CD11c+, which have not been previously described. The small CD14+CD11c+ subset also included low levels of CD4, consequently this could represent a different cell subset, e.g. monocytes or background and we did not explore this subset further.

However, the CD57+ cluster caught our attention. Interestingly, the CD57+ B cell cluster lacked differences in other expression markers. On other cells, CD57 is expressed on mature cytotoxic NK and on terminally differentiated T cells and theses subpopulations have been shown to be expanded in aging and persistent infections (326). So far, expression of CD57 on B cells has only been reported on specific B cell lymphomas (327,328). In order to confirm CD57 expression on B cells in SLE patients, we analyzed baseline sample from five of our SLE patient using flow cytometry and could confirm a small but visible expression of CD57 on CD20+ cells. To further confirm our dataset, we analyzed B cell subsets from multiple sclerosis (MS) patients which were run with the same panel and at the same mass cytometry facility. We could replicate CD57 expression on B cells in MS at baseline. The CD57+ B

cells in MS and SLE patients disappeared upon treatment with dimethyl fumarate therapy or belimumab. Given the expression of CD57 on differentiated T cells and NK cells, the association with age and persistent infection, as well as expression on lymphomas, one might speculate if they are part of the “atypical” B cell repertoire and might share similar features to ABCs.

Next, we analyzed well defined B cell phenotypes in response to belimumab treatment. As expected, B cell counts gradually decreased during follow-up. Lower B cell counts were discovered in patients which reached lupus low disease activity after 24 months of BAFF inhibition. Furthermore, we found that patients with higher B cell counts at baseline were less likely to respond to belimumab therapy and to reach the lupus low disease activity.

A rapid decline of naïve B cells and ABCs could be observed after 3 months of belimumab treatment with a continuous reduction over time. The CD57+ population behaved similarly to ABCs in response to BAFF inhibition. BAFF receptors have been reported on ABCs but their survival seemed not to be restricted to BAFF in mice (197,329).
Interestingly, we could observe a rapid and remaining reduction in ABC-numbers in early responders while ABCs in late responders decreased more gradually. In non-responder’s ABCs seemed to be unaltered highlighting the potential pathogenetic role of ABCs in SLE. However, it remains unclear, why ABCs respond differently to BAFF inhibition in patients.

Double negative (DN) B cells decreased more gradually over time and seemed to reach a plateau at 12 months which is aligned with previous reports (277). Unfortunately, we did not examine the different DN subsets. Given the previous studies about DN2 cells and ABCs being part of DN cells it would be of great interest to further dissect this population.

Switched memory B cells seem to be more resistant to BEL treatment. After an initial expansion, memory B cells decreased non-significantly after 12 months of BAFF inhibition and returned to baseline after 24 months. Similar results have been demonstrated previously and memory B cell survival has been shown to be independent from BAFF and APRIL (277,330). Noteworthy, when we compared surface expression levels between SLE responder index rates vs early responders, IgA+ expression was altered in late responders and we found prominent differences in IgA+ CD27+ B cells in early vs late responders. This is interesting since humans presenting mutations within the *BAFF-R* gene have lower levels of IgG and IgM levels but not IgA, suggesting that IgA producing cells might not be dependent

on BAFF (331). However, our results might suggest that only certain IgA specific cells are independent of BAFF.

Plasmablasts counts were not altered in PB by belimumab therapy even though previous studies indicated a decrease of plasmablasts at later timepoints (277,278). Jacobi et al. reported a gradual decline in total IgM-producing plasmablasts but not in IgG-producing cells (277). The survival of plasmablasts is probably maintained by circulating APRIL, which is not inhibited by belimumab (277,330,332).

Subsequently, we were interested in the correlation between the expression levels of individual markers and the time of treatment. Thus, we analyzed alterations of cell surface antigen expression after 0-3 months (early), 3-6 months (intermediate), and 6-36 months (late) of treatment. It is noteworthy, that the expression levels did not necessary represent changes in B cell subsets but could indicate a relative increase in expression of these markers. The expression levels changed primarily shortly after initializing treatment, while only a few markers changed expression during later time points. Interestingly, CD38 initially decreased, however, after 6-36 months increased. Performing a paired marker analysis, we explored the relationship between markers over early, intermediate and late timepoints. This allowed us to link the individual expression of surface markers to B phenotypes. CD38 was co-expressed with CD21 and CD22, in early timepoints, but oppositely expressed in later timepoints. This observation resulted from the depletion of native B cells while transitional type 1 B cells (CD38+IgM+IgD+CD22-CD21-CD27-) were resilient to belimumab treatment. An increased ratio of transitional B cells was reported in individuals with a deleted BAFF-R gene, suggesting that transitional B cells are BAFF independent (331).

The numbers of monocytes, T helper and cytotoxic T cells were not significantly affected by the treatment. Nor could we observe any significant correlation between expression levels of markers on T cells and BAFF treatment over time. However, certain BAFF-R have been described on T cell subsets. TACI was found on activated T cells (333,334) and elevated BR-3 expression was described on T_{FH} cells of SLE patients(335). Hence, a detailed analysis of T cell subtypes upon belimumab treatment would be of great interest in the future.

Correlating our immunological findings with low disease activity, we observed that patients showing lupus low disease activity at 24 months displayed a lower number of naïve cells and switched, memory B cells at baseline in comparison to non-responders while the count of

plasmablast and DN cells did not differ. Furthermore, decreased CD11c+ expression positively correlated with clinical improvement. Disease activity and anti-dsDNA antibodies decreased rapidly following belimumab treatment. While disease activity continued to decrease during follow-up, anti-dsDNA did not. Both correlated with early immunological alterations but not with later changes. This effect could result from a possible depletion of follicular B cells, marginal zone B cells or short-lived plasma cells which have been proven to be BAFF dependent (336). However, ABCs could also play an important role. ABCs are correlated with anti-dsDNA titers, are able to quickly differentiate into antibody-secreting cells and exhibited autoreactivity (185,200,337).

In summary, we observed a rapid decrease of B cell numbers in earlier stages of development in response to belimumab treatment while late-stage B-cell subsets are more robust and were affected later in a more gradual phase. SLE activity showed a rapid and continues decrease after belimumab treatment. ABCs decreased rapidly after treatment in early responders. However, in late responders, ABCs seemed more resilient against BAFF inhibition. IgA+ memory B cells showed a similar pattern as ABCs. Overall high B cell counts predicted unfavorable treatment outcomes, highlighting the importance of testing B-cell counts prior to belimumab treatment.

4.4 SYNOVIAL IL-6 AND IL-17A CYTOKINE SIGNATURE IN LUPUS ARTHRIIS

Arthritis is a frequent clinical manifestation in SLE patients (227). Yet, little attention has been given to investigating the pathogenicity of lupus arthritis. In **Paper III,** we explored the inflammatory environment in the affected joints of SLE patients. Hence, we examined T cell associated cytokines in synovial fluid (SF) samples from 17 SLE patients in comparison with matched serum samples and RA-SF samples (n=10). Here, we discovered that the two cytokines IL-17A and IL-6 were abundant in SF of SLE patients.

Elevated serum IL-6 concentrations have been associated with SLE, correlated with disease activity and have been reported in inflamed tissue, such as kidney of lupus nephritis and in cerebrospinal fluid of lupus psychosis patients (203,338,339). Our study has now demonstrated increased levels of IL-6 in SF of SLE patients with arthritis. IL-6 is a cytokine which is released in acute response. Moreover, in the context of the synovial joint in SLE, IL-6 might have a pathogenic role of in mediating local inflammation and damage in the affected tissues (340).

A previous study by Eilertsen et al. found an association between serum IL-6 levels, arthritis and joint deformation in SLE patients (232). In our dataset, although IL-6 concentrations were significantly higher in SF compared to serum, serum IL-6 correlated with IL-6 levels from SF *(r=0.7, p=0.0001).* Additionally, IL-6 was also increased in SF from RA patients, which is in line with previous findings linking increased IL-6 to chronic synovitis (341). During inflammation, IL-6 fulfils several different functions locally and systemically. It affect many different cells such as T cells, B cells, synovial fibroblasts and hepatocytes by promoting cell differentiation and inducing synthesis of cytokines and acute phase proteins, such as CRP (340,342,343).

Similar to IL-6, IL-17A concentrations in serum of SLE patients have been associated with disease activity and IL-17A-producing T cells have been identified in kidney of SLE patients (344,345). IL-17A can recruit monocytes and neutrophils at the site of inflammation (346). In SF of lupus arthritis patients, IL-17A was abundant and we could observe a weak correlation between IL-6 and IL-17A (r=0.39, *p=0.03*, CI=0.02-0.66) but not in between serum IL-17A concentrations. IL-6 and IL-17A are closely interlinked. Recently, it has been shown that IL-17A can induce production of IL-6 by monocytes and DC (57,58). Furthermore, together with TGF, IL-23 and IL-1 beta, IL-6 is able to induce differentiation of naïve T cells into the Th17 phenotype (342,343).

Interestingly, IL-17A concentrations seemed to cluster into two groups: low (<80pg/ml) and high concentration (>200pg/ml). However, we did not find any significant differences in age, disease duration, clinical symptoms, therapy nor serological features which could have led to this division. Our study was conducted retrospectively, on a relatively small heterogenic patient cohort. Information of disease activity and systematic assessment of joint at the time of sampling were lacking and are a major limitation of our study. Thus, we were limited in assessing clinical associations and future studies need to be done in order to address this issue.

Besides IL-17A and IL-6, the cytokine assay comprised the cytokines IFN-γ, TNF, IL-2, IL-4 and IL-10. In 41% of SLE patients, low concentrations of IL-10 were detected in SF and serum while low levels of IL-4 and IL-2 could be primarily found in sera. IFN-γ was found in four SLE patients in very low concentrations while TNF levels were below the limit of detection in all of the samples. Surprisingly, TNF was also not detected in RA-SF patients, which normally exhibit high levels of TNF in SF (349). However, many of them were on TNF inhibitors at the

time of sampling. Due to the low sample size and the use of only one method, it is difficult to conclude if these cytokines are not present in SLE-SF or if the method was not adapted to detect them.

Next, we investigated the source of cytokines and explored the cellular compartments in SF of SLE patients. Since mononuclear cells in SF of SLE patients are low, we could only retrieve three SFMCs samples from two SLE patient, one with two different timepoints. The cells were analyzed for T cell phenotypes and if possible, for B, dendritic cell and monocyte subsets using multicolor flow cytometry. Due to the low sample size, we were not able to perform any statistical test and our findings are more of exploratory nature. Future studies have to be performed in order to confirm our results from these two patients.

Autoantibodies play an essential role in the pathology of SLE patients and phenotypic B cell abnormalities are common in SLE patients (349). Interestingly, in SF of SLE patients, CD19+ B cells were rare (<1%). The small fraction of B cells in SF exhibited a memory phenotype, for one patient containing age/autoimmune associated (ABC) CD11c+ B cells. A lower frequency of B cells in SF compared to PB has been reported in RA patients although it was higher than our findings in SLE patients (68). In RA, RF and ACPA titers are increased in SF and a higher frequency of ACPA-IgG secreting cells has been demonstrated in SF of RA patients (68,350). Thus, we investigated the presence of rheumatoid factor (RF) in the cohort of SLE patients in SF and serum. Only two patients were positive for RF and exhibited lower levels in SF compared to sera. Previous studies reported the presence of ANA antibodies in SF, however the exact frequency is unknown (228). These finding suggest that B cells either do not to accumulate in SF or stay in the synovial tissue. However, future studies have to confirm this theory and it would be of great importance to investigate synovial tissue biopsies of SLE patients for ectopic germinal centers.

IL-6 is produced by monocytes, dendritic cells, fibroblasts and epithelial cells and less frequently by lymphocytes (340). Consequently, we were interested in monocyte and dendritic cell subsets in SF. We found that 64-67% of HLA-DR+ cells in SLE-SF were classical monocytes (CD14+ CD16-). A study in ST- segment elevation myocardial infarction patient revealed that IL-17A is able to induce the production and secretion of IL-6 from CD14+CD16- and CD14+CD16+ monocytes (351). Approximately 30% of HLA-DR+ cells in SLE-SF were non-monocyte cells and consisted predominantly of classical DC (CD11c+, cDC). The frequency of plasmacytoid DC as well as the subtypes of cDCs varied

between patients. In patient one, cDC consisted largely of cDC1 while the other displayed equal proportions of cDC1 and cDC2. cDC2 have been suggested to play an important role in inducing Th17 differentiation in the intestine through IL-6 production (352).

IL-6 is not only able to promote polarization of T cells into Th17 cells but is also involved in the differentiation of T follicular helper, as well as cytotoxic CD8+ T cells (340). In our study, the frequency of CD8+ cell within the CD3+ compartment was enriched in SF of SLE patients compared to PB. EOMES and Granzyme A (GZMA) were expressed by ~70-80% of CD8+ T cells. EOMES is an essential transcription factor for CD8+ T cell memory and effector differentiation (353) regulating *PRF1* (Perforin-1) transcription (94). An enrichment of EOMES+, GZMA+ CD4+ T cells could also be detected in SF of SLE patients. In RA-SF, a fraction of EOMES+CD4 T cells expressed GZMB and perforin-1, which is a sign for a cytotoxic phenotype. GZMA has been shown to stimulate IL-6, IL-8, and TNF- α production by human PBMC and monocytes (354). Our group recently identified an increased frequency of EOMES+ CD4+ cells in healthy donors homozygous for the *PTPN22* risk allele (1858T) as well as in synovial fluid of RA (355). *PTPN22* risk allele (1858T) also associates with SLE and might contribute to this phenotype in SLE (356).

We could also identify that a high proportion of CD4 and CD8 T cells were PD-1+ HLA-DR+/- in all SF samples. PD-1 is upregulated in activated T cells and an increase of PD-1 in synovial fluid T cells has been reported in SF in other rheumatic diseases (354,357). Interestingly, we could identify a distinct population of PD-1^{high} HLA-DR^{high} T cells within the CD4+ PD-1+ subset. PD-1^{high} HLA-DR^{high} ,so called T peripheral helper cells, have been described in SF and synovium of ACPA+ RA patients (322). T_{PH} cells are able to promote B cell responses and antibody production. In SLE, T_{PH} cells can be found in the kidneys of LN patients and correlated with disease activity (217,323).

The frequency of follicular helper T cells (Tfh, PD1+ CXCR5+) was also increased in SF in both SLE patients while Th1 cell frequencies (CXCR3+) were comparable between SF and PB. In ACPA+ RA patients majority of T cells express CXCR3+ in SF (358). Regulatory T cells (Tregs, CD25+ FoxP3+) are increased in SF and is in line with previous findings from other inflammatory arthritis (358).

A majority (35-55%) of CD4+ T cells expressed the surface molecule CCR6, a marker for the Th17 cell subset (359). Th17 cells have been previously linked with SLE, other rheumatic

diseases and inflammatory arthritis. An enrichment of CCR6+ CD4+ cells as well as Th17 cells have been described in the affected joint and peripheral blood of psoriatic arthritis patients (360). In SLE, Th17 cells have been shown to be enriched during SLE flare and diminish after effective treatment (361,362). Previous studies have shown that IL-17 producing CD4+ cells are primarily constrained to CCR6 (363). In order to investigate, if SF-derived T cells are capable of producing IL-17A, we stimulated SFMCs (n=1) *in-vitro* with anti-CD3/CD28 beads. Intracellular IL-17A and IFN-γ production was analyzed using flow cytometry. IL-17A was produced by the CCR6+ T helper cell subset but was absent in non-CD4 expressing T cells. Nevertheless, other cells could still contribute to IL-17A production in SF as double negative, gamma delta T cells, innate lymphoid cells, neutrophils and mast cells have been recognized to produce IL-17A in psoriatic arthritis patients (292). We only investigated the production of T cells and the type of stimulation might have influenced the capacity of CD8+ cells to produce IL-17A. A recent study discovered that stimulation with CD3/CD28 beads did not stimulate CD8+ cells to produce IL-17A in SFMCs from psoriatic arthritis patients (363).

In addition to IL-17A, we also studied the ability of SF-derived T cells to produce IFN-γ. Th17 have been reported to be a plastic population, which can adopt functions from other T helper cells (207). Th17 can adept Th1 features and produces IL-17A and IFN-γ simultaneously. Th17/Th1 cells express CCR6 and CXCR3 (364,365), in SF of SLE patients, CXCR3+ CCR6+ were not enriched and IFN-γ production was only observed in IL-17 negative CD4+ T cells. However, we did observe an abundant production of IFN-γ in CD4+ and CD8+ T cells, suggesting that IFN-γ might play an important role in SF of SLE patients.

CCR4 which is predominately expressed on Th2 cells, can be found on a proportion of Th17 cells, however without the production of Th2 associated cytokines and CCR4 might rather play a role in trafficking (365–367). Here, we found that CCR4 was increased on CD4+ cells in SF of SLE patients and around 40% of the CCR6+ T cells co-expressed CCR4. It is also noteworthy that 30% of T_{PH} cells co-expressed CCR6, which highlights the plasticity of these cells.

It is important to mention, that our study is rather exploratory and future research needs to replicate our results. The main limitations are the low sample size, especially in the cell analysis (n=2) and the lack of clinical data including antibody titers, joint involvements and diseases activity. Additionally, the patients in our cohort exhibited a long disease duration and history of treatments, which could have influenced our cytokine and cellular data. Hence,

further investigations in patients with shorter disease duration, less exposure to treatment would be required and it would be of great interest to analyze synovial tissue, to investigate the presence of ectopic germinal centers.

Overall, our study explored the immunology underlaying lupus arthritis and suggests an involvement of IL-17 as well as a pathogenic role of IL-6 in the affected joint. The presence of CCR6+ CD4+ T cells which are able to produce IL-17A hints towards an involvement of Th17 cells in synovial fluid. GZMA+ EOMES+ CD4+ and CD8+ T cells are abundant and could contribute to the inflammatory milieu in SF of SLE patients. Yet, future research has to determine if those cells are of cytotoxic phenotype or fulfill other functions in synovial fluid of SLE patients. Lastly, we could identify T_{PH} cells in SF of SLE patients, which have been shown to promote B cell responses in SLE. B cells were rare in SF of SLE patients; however, they might be present in synovial tissue of inflamed joints. Altogether, our findings indicate a contribution of T cells in pathogenesis of arthritis in SLE patients. This suggests that lupus arthritis patients may improve with IL-17A or IL-6 inhibitor

4.5 CHARACTERISTICS OF CITRULLINE-SPECIFIC B CELLS IN RA PATIENTS

ACPAs are associated with more severe disease activity. Even though extensive research has been conducted on ACPAs, the contribution of ACPA to the pathogenesis of arthritis remains still unsolved and little is known about the anti-citrulline reactive B cells. To answer some of these questions in **Paper IV**, we made use of a tetramer enrichment strategy and combined these tetramers with single-cell cloning technologies in order to identify and study rare citrulline-reactive B cell clones in RA patients.

First, we constructed fluorescently labeled peptide tetramers from two previously described antigens: citrullinated human filaggrin (Cfc1/CCP1) and alpha-enolase (CEP-1) **(Figure 9).** Subsequently, the constructs were used to detect citrulline-reactive B cells in peripheral blood (PB) of seropositive and seronegative RA patients as well as healthy individuals. Interestingly, we could detect a small proportion of citrulline-reactive B cells in PB of healthy controls where the majority of citrulline-reactive B cells displayed a naïve phenotype (IgD+CD27-). Similar results were observed in seronegative RA patients. In the contrary, in PB of seropositive RA patients, citrulline-reactive B cells were enriched in particular in the switched memory compartment (IgD- CD27+) and the frequency of switched citrulline specific B cells correlated with antibody titers and disease activity score. These findings are in line with previous results (368).

In order to obtain information about the somatic hypermutation, Ig gene rearrangements, origin and binding specificities of citrulline reactive B cells, single cell heavy and light chain sequences were obtained from single citrulline specific B cells. Therefore, we used nested polymerase chain reaction (PCR) amplifications or high throughput RNA-sequencing. As a control, Immunoglobulin heavy (IgH) and immunoglobulin light (IgL) sequences from single random blood switched memory B cells were simultaneously amplified and analyzed. Furthermore, we cloned and expressed recombinant monoclonal antibodies from paired B cell receptor (BCR) sequences and analyzed them for citrulline reactivity using ELISA and peptide arrays.

The sequence analysis revealed a biased variable (V) gene usage and complementarity-determining regions 3 (CDR3) length in heavy and light chain of the captured citrulline specific B cell which was primarily absent in the random switched memory compartment. Interestingly, the gene rearrangements were more diverse in the CEP-1 specific B cells. Additionally, citrulline tetramer binding B cells exhibited extensive somatic hypermutation which was concentrated to the CDR regions. The average replacement mutation to silence ratio was also increased which is a sign for a positive selection pressure. Our group demonstrated comparable results in APCA+ B cells originating from SF of RA patients, with a higher mutation frequency and biased V gene usage in ACPA+ B cells compared with ACPA- B cells (49).

Next, we used the generated sequences to assess the phylogenetic relationships of enlarged heavy and light chain linages. This revealed that the expanded clades originated from a small number of unmutated precursor B cells and the clonal divergency occurred as a result of somatic hypermutations. Interestingly, further analysis of the BCRs from tetramer captured B cells showed that mAb originating from expanded B cell clades were more likely to bind citrulline specific epitopes than non-clade-derived mAb. Additionally, citrulline reactive mAb (ACPAs) displayed a citrulline dependent multi-reactivity to several peptide epitopes and full-length proteins. Several of the generated ACPAs exhibited a broad reactivity against citrullinated nuclear antigens and several were investigated further by our group and collaborators. Two of our ACPAs (37CEPT2C04, 37CEPT1G09) were explored in detail due to their nuclear binding properties and were observed to bind apoptotic cells from humans and mice as well as NETs. Moreover, their reactivity appears to be primarily directed towards histones(369). Sahlström et al. have shown that monoclonal ACPAs bind to distinct consensus peptide motifs which are present on various proteins which explains the difference in binding patterns and multi-specificity towards citrullinated peptides and proteins(369).

Lastly, we investigated the pathogenic role of four of our generated mAb *in-vitro*. Previously studies reported that ACPAs alone are not able to induce arthritis but enhance tissue injury in murine experimental arthritis models(47,69). Hence, we made use of a transient joint inflammation model. For this experiment, mice were injected intravenously with murinized mAb and 8 days later with LPS in the ankle joint of the left hindpaw. Sensitivity to mechanical stimulation was determined and inflammation was scored visually (0-3 scale). Two citrulline polyreactive mAbs were able to induce pain-like behavior and increased periarticular immune cell infiltration. In contrast, the control antibodies showed a typical course of acute arthritis resolution, highlighting the potential pathogenic effect and the functional diversity of ACPAs.

Overall, we utilized the tetramer enrichment technology in order to characterize rare citrulline specific B cells in blood of seropositive RA patients. Our data suggests that a few autoreactive B cell clones undergo somatic hyper mutation and positive selection with the help of T cells and GC reactions which can lead to a diverse autoreactive BCR repertoire. Further studies in our group revealed that ACPAs are a functionally diverse subset of autoantibodies which recognize distinct consensus peptide motifs present on various proteins which leads to multi-reactivity (48,370). In addition, the generated mAb were and are currently used to investigate *in-vivo* effect of citrulline reactive B cells.

5 CONCLUSIONS AND OUTLOOK

Paper I:

- Frequency of age/autoimmune-associated B cells decreases after ritxuimab treatment
- T follicular helper cell frequencies are unaffected while PD-1^{high} CD4+ T cell frequency decreases

Paper II:

- SLE patients exhibit a pronounced B cell differentiation.
- Belimumab rapidly effects early B cell development stages while late stage B cell stages gradually alter in later time points or remain uneffected
- B cell counts, CD11c+ and IgA expression correlate with clinical outcomes

Paper III:

- Elevated levels of IL-6 and IL-17A are found in SF of SLE patients
- Enrichment of Th17, T pheripheral helper and EOMES+ T cells is observed in SF of SLE patients

Paper IV

- Citrulline reactive B cells are mainly of a memory phenotype in seropositive RA patients
- The diverse citrulline reactive B cell repertoire originated from a few unmutated B cell clones through several rounds of somatic hypermutation and positive selection
- mAb generated from citrulline reactive B cells are highly multi-reactive, display different binding patterns and are able to promote pain and cell infiltration in a urine experimetnal arhtitis model.

B cell dysfunction and abnormalities are frequent in SLE patients. They display a more differentiated B cell repertoire with enrichment of age/autoimmune-associated B cells, switched memory B cells and plasmablasts. Defects in peripheral and central tolerance have also been described in the disease including GC reactions as well as the accumulation of polyreactive and self-reactive immature and naïve B cells. Both mechanisms are likely to contribute to the disease pathogenesis (162–164). Thus, B cell depleting therapy seemed to be a promising tool for the treatment of SLE and other autoimmune diseases. In order to understand the effects and clinical outcomes it is of great importance to study the influence of B cell depleting therapy on different cell phenotypes, autoantibody titers and cytokine levels.

Rituximab and belimumab both rapidly reduce the numbers of B cells in the earlier phases of development (transitional, unswitched-memory, naïve B cells). While switched memory B cells are more resilient towards belimumab and reduce more gradually, they are depleted with rituximab. However, it seems that specific memory B cells are less affected by rituximab therapy, since the remaining B cells are of a memory or plasmablast phenotype and a higher fraction of memory B cells are related to earlier relapses (241,262,263). It is still surprising that B cell depleting therapy did only show modest or even no success in SLE patients in clinical trials (246,247,268). This result could be due to the lack or delay for depletion of late-stage B cells or that B cells in the tissue are more resilient to the treatment. However, at the same time, it also highlights that some patients can improve in the disease even without plasma cell depletion nor change in the autoantibody levels, highlighting the heterogenicity and complexity of the disease.

In our studies, both B cell depleting therapies were able to reduce age/autoimmune-associated B cells (ABCs). So far, we were not able to correlate the clinical outcome with the ABC frequency after rituximab treatment. In patients with belimumab therapy, decreased CD11c expression correlated with clinical improvement, and non-responder seem to exhibit a more resilient population of ABCs compared to responders. It is noteworthy, that the anti-dsDNA antibody concentration only changed in the first month, when memory and plasma blast levels were still rather stable, suggesting the importance of ABCs. ABCs have been shown to be enriched of autoreactive BCRs, e.g. in dsDNA binding BCRs. Still, it remains unclear, why the number of ABCs decrease rapidly in responders and why they seem more resilient in non-responders. Interestingly, high B cell counts are a predictor for shorter clinical response upon belimumab treatment in general. The data suggests that a combination of rituximab and belimumab could be beneficial in these patients. Indeed, a successful combination of belimumab and rituximab has been reported in small case control studies, and clinical trials are on their way (NCT03312907, NCT02260934) (371,372). These clinical trials were initiated due to the fact that, after treatment with rituximab, patients exhibit higher levels of BAFF. High BAFF levels are associated with shorter clinical response and earlier relapses in patients treated with rituximab (265–267). A combination of these two treatments might also be valuable for patients with high B cell counts.

The question arises: “Are we targeting the right cells and subsets in SLE?” More effective targeting of ABCs and plasma cells may be an effective therapeutic strategy for SLE. However, autoimmune diseases result from a complex interplay between the innate and the

adaptive immune system. Moreover, SLE is highly heterogenous disease with different clinical manifestations and immune pathways involved. A personalized approach based on the study of clinical manifestations in combination with specific laboratory measurements, such as cytokines and cell subsets, could be a useful tool for the clinicians to decide the best treatment strategies for each patient. Therefore, it is crucial to understand the pathogenic mechanisms and compounds involved in the different manifestations, disease mechanisms and patient subgroups.

Lupus arthritis is a common clinical manifestation in SLE and influence the life quality of the patients (227,373). Little is known about the pathogenic mechanisms of the disease. For that reason, we analyzed SF samples from SLE patients and found elevated levels of IL-6 and IL-17A as well as an enrichment of Th17, T peripheral helper cells and cytotoxic T cells in SF of SLE patients. Although further studies, with a higher sample size and more in-depth analysis of the cytokine profile and cells involved are needed in order to confirm our findings, they still suggest a potential therapeutic role of IL-6 and IL-17A in lupus arthritis patients. Indeed, IL-6 inhibition has been demonstrated to improve arthritis in lupus arthritis patients(374). Additionally, IL-17 inhibition seems a promising target for some SLE patients, including the potential of IL-17 to promote the survival of long lived plasma cells and a clinical trial is currently performed in SLE patients (NCT03866317,(375).

In the future, could we detect and deplete only autoreactive B cells in the patient? Nowadays, B cell tetramers are frequently used to analyze antigen specific B cells and are a useful tool to study the B cell repertoires, the origin and characteristics of autoreactive B cells in autoimmune patients. Using citrulline specific tetramers, we were able to detect and analyze citrulline reactive B cells in blood of RA patients. Our study revealed that citrulline reactive B cells develop from a restricted number of B cells which underwent several rounds of somatic hypermutations. This expansion might be driven by citrulline reactive T cells and starts before the development of clinical symptoms. The extensive somatic hypermutation and presence of ACPA+ B cells hints towards a continuously active B cell response. How exactly joint inflammation and RA is triggered is still unknown. ACPAs alone cannot induce arthritis in mice but mAbs originating from tetramer captured B cells were able to promote joint inflammation and pain of mice, implicating that they are indeed important in the pathogenicity of RA. Further studies with these antibodies demonstrated that ACPAs have different binding patterns, functions and might originate from different immune responses. Recently, our unit demonstrated, that ACPAs, which bind nuclear antigens, might develop

independently from the citrullinating enzyme PAD through neutrophil activation and NET formation (369), suggesting that several mechanism can result in a diverse set of ACPAs. However, it is still not exactly known how citrulline reactive B cells are able to escape the tolerance checkpoints.

To conclude, rheumatic diseases are highly heterogenous with different pathogenic mechanisms. Autoantibody production starts before the onset of clinical symptoms and can be found in some healthy individuals. Genetic and environmental factors probably contribute to the development of autoreactive antibody as well as to the break of tolerance.
Questions are still remaining:" How tolerance is broken in different individuals? Would it be possible to restore tolerance mechanisms in these patients?" These are questions which hopefully future research will answer. For now, I think we would already improve clinical diagnostic and treatment if we chose therapeutic agents on individual bases. This strategy would be based on an in-depth integration of cellular markers, cytokines, complement and autoantibody profile together with clinical manifestations.

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